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(54) T7 DNA polymerase.

(57) This invention relates to T7-type DNA polymerases and methods for using them including a method for determining the nucleotide base sequence of a DNA molecule, comprising annealing said DNA molecule with a primer molecule able to hybridize to said DNA molecule; incubating separate portions of the annealed mixture in at least four vessels with four different deoxynucleoside triphosphates, a processive DNA polymerase, wherein said polymerase remains bound to said DNA molecule for at least 500 bases before dissociating in an environmental condition normally used in the extension reaction of a DNA sequencing reaction, said polymerase having less than 500 units of exonuclease activity per mg of said polymerase, and one of four DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base. The agent terminates at a different specific nucleotide base in each of the four vessels. The DNA products of the incubating reaction are separated according to their size so that at least part of the nucleotide base sequence of the DNA molecule can be determined.

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T7 DNA POLYMERASE

This invention relates to DNA polymerases suitable for DNA sequencing.

DNA sequencing involves the generation of four populations of single stranded DNA fragments having one defined terminus and one variable terminus. The variable terminus always terminates at a specific given nucleotide base (either guanine (G), adenine (A), thymine (T), or cytosine (C)). The four different sets of fragments are each separated on the basis of their length, on a high resolution polyacrylamide gel; each band on the gel corresponds colinearly to a specific nucleotide in the DNA sequence, thus identifying the positions in the sequence of the given nucleotide base.

Generally there are two methods of DNA sequencing. One method (Maxam and Gilbert sequencing) involves the chemical degradation of isolated DNA fragments, each labeled with a single radiolabel at its defined terminus, each reaction yielding a limited cleavage specifically at one or more of the four bases (G, A, T or C). The other method (dideoxy sequencing) involves the enzymatic synthesis of a DNA strand. Four

separate syntheses are run, each reaction being caused to terminate at a specific base (G, A, T or C) via incorporation of the appropriate chain terminating dideoxynucleotide. The latter method is preferred since the DNA fragments are uniformly labelled (instead of end labelled) and thus the larger DNA fragments contain increasingly more radioactivity. Further, ^{35}S-labelled nucleotides can be used in place of ^{32}P-labelled nucleotides, resulting in sharper definition; and the reaction products are simple to interpret since each lane corresponds only to either G, A, T or C. The enzyme used for most dideoxy sequencing is the Escherichia coli DNA-polymerase I large fragment ("Klenow"). Another polymerase used is AMV reverse transcriptase.

Summary of the Invention

In one aspect the invention features a method for determining the nucleotide base sequence of a DNA molecule, comprising annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating separate portions of the annealed mixture in at least four vessels with four different deoxynucleoside triphosphates, a processive DNA polymerase wherein the polymerase remains bound to a DNA molecule for at least 500 bases before dissociating in an environmental condition normally used in the extension reaction of a DNA sequencing reaction, the polymerase having less than 500 units of exonuclease activity per mg of polymerase, and one of four DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base. The agent terminates at a different specific nucleotide base in each of the four vessels. The DNA products of the incubating reaction are separated according to their size so that at least a part of the nucleotide base sequence of the DNA molecule can be determined.

In preferred embodiments the polymerase remains bound to the DNA molecule for at least 1000 bases before dissociating; the polymerase is substantially the same as one in cells infected with a T7-type phage (i.e., phage in which the DNA polymerase requires host thioredoxin as a subunit; for example, the T7-type phage is T7, T3, ΦI, ΦII, H, W31, gh-1, Y, A1122, or SP6, Studier, 95 Virology 70, 1979); the polymerase is non-discriminating for dideoxy nucleotide analogs; the polymerase is modified to have less than 50 units of exonuclease activity per mg of polymerase, more preferably less than 1 unit, even more preferably less than 0.1 unit, and most preferably has no detectable exonuclease activity; the polymerase is able to utilize primers of as short as 10 bases or preferably as short as 4 bases; the primer comprises four to forty nucleotide bases, and is single stranded DNA or RNA; the annealing step comprises heating the DNA molecule and the primer to above 65°C, preferably from 65°C to 100°C, and allowing the heated mixture to cool to below 65°C, preferably to 0°C to 30°C; the incubating step comprises a pulse and a chase step, wherein the pulse step comprises mixing the annealed mixture with all four different deoxynucleoside triphosphates and a processive DNA polymerase, wherein at least one of the deoxynucleoside triphosphates is labelled; most preferably the pulse step performed under conditions in which the polymerase does not exhibit its processivity and is for 30 seconds to 20 minutes at 0°C to 20°C or where at least one of the nucleotide triphosphates is limiting; and the chase step comprises adding one of the chain terminating agents to four separate aliquots of the mixture after the pulse step; preferably the chase

step is for 1 to 60 minutes at 30°C to 50°C; the terminating agent is a dideoxynucleotide, or a limiting level of one deoxynucleoside triphosphate; one of the four deoxynucleotides is dITP or deazaguanosine; labelled primers are used so that no pulse step is required, preferably the label is radioactive or fluorescent; and the polymerase is unable to exhibit its processivity in a second environmental condition normally used in the pulse reaction of a DNA sequencing reaction.

In other aspects the invention features a) a method for producing blunt ended double-stranded DNA molecules from a linear DNA molecule having no 3' protruding termini, using a processive DNA polymerase free from exonuclease activity; b) a method of amplification of a DNA sequence comprising annealing a first and second primer to opposite strands of a double stranded DNA sequence and incubating the annealed mixture with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase, preferably less than 1 unit, wherein the first and second primers anneal to opposite strands of the DNA sequence; in preferred embodiments the primers have their 3' ends directed toward each other; and the method further comprises, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the resulting DNA and incubating the annealed mixture with the polymerase; preferably the cycle of denaturing, annealing and incubating is repeated from 10 to 40 times; c) a method for <u>in vitro</u> mutagenesis of cloned DNA fragments, comprising providing a cloned fragment and synthesizing a DNA strand using a processive DNA polymerase having less than 1 unit of exonuclease activity per mg of polymerase; d) a method of producing active T7-type DNA polymerase from cloned DNA fragments under the control

of non-leaky promoters (see below) in the same cell comprising inducing expression of the genes only when the cells are in logarithmic growth phase, or stationary phase, and isolating the polymerase from the cell; preferably the cloned fragments are under the control of a promoter requiring T7 RNA polymerase for expression; e) a gene encoding a T7-type DNA polymerase, the gene being genetically modified to reduce the activity of naturally occurring exonuclease activity; most preferably a histidine (His) residue is modified, even more preferably His-123 of gene 5; f) the product of the gene encoding genetically modified polymerase; g) a method of purifying T7 DNA polymerase from cells comprising a vector from which the polymerase is expressed, comprising the steps of lysing the cells, and passing the polymerase over an ion-exchange column, over a DE52 DEAE column, a phosphocellulose column, and a hydroxyapatite column; preferably prior to the passing step the method comprises precipitating the polymerase with ammonium sulfate; the method further comprises the step of passing the polymerase over a Sephadex DEAE A50 column; and the ion-exchange column is a DE52 DEAE column; h) a method of inactivating exonuclease activity in a DNA polymerase solution comprising incubating the solution in a vessel containing oxygen, a reducing agent and a transition metal; i) a kit for DNA sequencing, comprising a processive DNA polymerase, defined as above, having less than 500 units of exonuclease activity per mg of polymerase, wherein the polymerase is able to exhibit its processivity in a first environmental condition, and preferably unable to exhibit its processivity in a second environmental condition, and a reagent necessary for the sequencing,

selected from a chain terminating agent, and dITP; j) a method for labelling the 3' end of a DNA fragment comprising incubating the DNA fragment with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase, and a labelled deoxynucleotide; k) a method for in vitro mutagenesis of a cloned DNA fragment comprising providing a primer and a template, the primer and the template having a specific mismatched base, and extending the primer with a processive DNA polymerase; and l) a method for in vitro mutagenesis of a cloned DNA fragment comprising providing the cloned fragment and synthesizing a DNA strand using a processive DNA polymerase, having less than 50 units of exonuclease activity, under conditions which cause misincorporation of a nucleotide base.

This invention provides a DNA polymerase which is processive, non-discriminating, and can utilize short primers. Further, the polymerase has no associated exonuclease activity. These are ideal properties for the above described methods, and in particular for DNA sequencing reactions, since the background level of radioactivity in the polyacylamide gels is negligible, there are few or no artifactual bands, and the bands are sharp -- making the DNA sequence easy to read. Further, such a polymerase allows novel methods of sequencing long DNA fragments, as is described in detail below.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Figs. 1-3 are diagrammatic representations of the vectors pTrx-2, mGP1-1, and pGP5-5 respectively;

Fig. 4 is a graphical representation of the selective oxidation of T7 DNA polymerase;

Fig. 5 is a graphical representation of the ability of modified T7 polymerase to synthesize DNA in the presence of etheno-dATP; and

Fig. 6 is a diagrammatic representation of the enzymatic amplification of genomic DNA using modified T7 DNA polymerase.

Fig. 7, 8 and 9 are the nucleotide sequences of pTrx-2, a part of pGP5-5 and mGP1-2 respectively.

Fig. 10 is a diagrammatic representation of pGP5-6.

DNA Polymerase

In general the DNA polymerase of this invention is processive, has no associated exonuclease activity, does not discriminate against nucleotide analog incorporation, and can utilize small oligonucleotides (such as tetramers, hexamers and octamers) as specific primers. These properties will now be discussed in detail.

Processivity

By processivity is meant that the DNA polymerase is able to continuously incorporate many nucleotides using the same primer-template without dissociating from the template, under conditions normally used for DNA sequencing extension reactions. The degree of processivity varies with different polymerases: some incorporate only a few bases before dissociating (e.g. Klenow (about 15 bases), T4 DNA

polymerase (about 10 bases), T5 DNA polymerase (about 180 bases) and reverse transcriptase (about 200 bases) (Das et al. J. Biol. Chem. 254:1227 1979; Bambara et al., J. Biol. Chem 253:413, 1978) while others, such as those of the present invention, will remain bound for at least 500 bases and preferably at least 1,000 bases under suitable environmental conditions. Such environmental conditions include having adequate supplies of all four deoxynucleoside triphosphates and an incubation temperature from 10°C-50°C. Processivity is greatly enhanced in the presence of *E. coli* single stranded binding (*ssb*), protein.

With processive enzymes termination of a sequencing reaction will occur only at those bases which have incorporated a chain terminating agent, such as a dideoxynucleotide. If the DNA polymerase is non-processive, then artifactual bands will arise during sequencing reactions, at positions corresponding to the nucleotide where the polymerase dissociated. Frequent dissociation creates a background of bands at incorrect positions and obscures the true DNA sequence. This problem is partially corrected by incubating the reaction mixture for a long time (30-60 min) with a high concentration of substrates, which "chase" the artifactual bands up to a high molecular weight at the top of the gel, away from the region where the DNA sequence is read. This is not an ideal solution since a non-processive DNA polymerase has a high probability of dissociating from the template at regions of compact secondary structure, or hairpins. Reinitiation of primer elongation at these sites is inefficient and the usual result is the formation of bands at the same position for all four nucleotides, thus obscuring the DNA sequence.

Analog discrimation

The DNA polymerases of this invention do not discriminate significantly between dideoxy-nucleotide analogs and normal nucleotides. That is, the chance of incorporation of an analog is approximately the same as that of a normal nucleotide or at least incorporates the analog with at least 1/10 the efficiency that of a normal analog. The polymerases of this invention also do not discriminate significantly against some other analogs. This is important since, in addition to the four normal deoxynucleoside triphosphates (dGTP, dATP, dTTP and dCTP), sequencing reactions require the incorporation of other types of nucleotide derivatives such as: radioactively- or fluorescently-labelled nucleoside triphosphates, usually for labeling the synthesized strands with ^{35}S, ^{32}P, or other chemical agents. When a DNA polymerase does not discriminate against analogs the same probability will exist for the incorporation of an analog as for a normal nucleotide. For labelled nucleoside triphosphates this is important in order to efficiently label the synthesized DNA strands using a minimum of radioactivity. Further, lower levels of analogs are required with such enzymes, making the sequencing reaction cheaper than with a discriminating enzyme.

Discriminating polymerases show a different extent of discrimination when they are polymerizing in a processive mode versus when stalled, struggling to synthesize through a secondary structure impediment. At such impediments there will be a variability in the intensity of different radioactive bands on the gel, which may obscure the sequence.

Exonuclease Activity

The DNA polymerase of the invention has less than 50%, preferably less than 1%, and most preferably less than 0.1%, of the normal or naturally associated level of exonuclease activity (amount of activity per polymerase

molecule). By normal or naturally associated level is meant the exonuclease activity of unmodified T7-type polymerase. Normally the associated activity is about 5,000 units of exonuclease activity per mg of polymerase, measured as described below by a modification of the procedure of Chase et al. (249 J. Biol. Chem. 4545, 1974). Exonucleases increase the fidelity of DNA synthesis by excising any newly synthesized bases which are incorrectly basepaired to the template. Such associated exonuclease activities are detrimental to the quality of DNA sequencing reactions. They raise the minimal required concentration of nucleotide precursors which must be added to the reaction since, when the nucleotide concentration falls, the polymerase activity slows to a rate comparable with the exonuclease activity, resulting in no net DNA synthesis, or even degradation of the synthesized DNA.

More importantly, associated exonuclease activity will cause a DNA polymerase to idle at regions in the template with secondary structure impediments. When a polymerase approaches such a structure its rate of synthesis decreases as it struggles to pass. An associated exonuclease will excise the newly synthesized DNA when the polymerase stalls. As a consequence numerous cycles of synthesis and excision will occur. This may result in the polymerase eventually synthesizing past the hairpin (with no detriment to the quality of the sequencing reaction); or the polymerase may dissociate from the synthesized strand (resulting in an artifactual band at the same position in all four sequencing reactions); or, a chain terminating agent may be incorporated at a high frequency and produce a wide variability in the intensity of different fragments in a sequencing gel. This happens because the frequency of

incorporation of a chain terminating agent at any given site increases with the number of opportunities the polymerase has to incorporate the chain terminating nucleotide, and so the DNA polymerase will incorporate a chain-terminating agent at a much higher frequency at sites of idling than at other sites.

An ideal sequencing reaction will produce bands of uniform intensity throughout the gel. This is essential for obtaining the optimal exposure of the X-ray film for every radioactive fragment. If there is variable intensity of radioactive bands, then fainter bands have a chance of going undetected. To obtain uniform radioactive intensity of all fragments, the DNA polymerase should spend the same interval of time at each position on the DNA, showing no preference for either the additon or removal of nucleotides at any given site. This occurs if the DNA polymerase lacks any associated exonuclease, so that it will have only one opportunity to incorporate a chain terminating nucleotide at each position along the template.

Short primers

The DNA polymerase of the invention is able to utilize primers of 10 bases or less, as well as longer ones, most preferably of 4-20 bases. The ability to utilize short primers offers a number of important advantages to DNA sequencing. The shorter primers are cheaper to buy and easier to synthesize than the usual 15-20-mer primers. They also anneal faster to complementary sites on a DNA template, thus making the sequencing reaction faster. Further, the ability to utilize small (e.g., six or seven base) oligonucleotide primers for DNA sequencing permits strategies not otherwise possible for sequencing long DNA fragments.

For example, a kit containing 80 random hexamers could be generated, none of which are complementary to any sites in the cloning vector. Statistically, one of the 80 hexamer sequences will occur an average of every 50 bases along the DNA fragment to be sequenced. The determination of a sequence of 3000 bases would require only five sequencing cycles. First, a "universal" primer (e.g., New England Biolabs #1211, sequence 5' GTAAAACGACGGCCAGT 3') would be used to sequence about 600 bases at one end of the insert. Using the results from this sequencing reaction, a new primer would be picked from the kit homologous to a region near the end of the determined sequence. In the second cycle, the sequence of the next 600 bases would be determined using this primer. Repetition of this process five times would determine the complete sequence of the 3000 bases, without necessitating any subcloning, and without the chemical synthesis of any new oligonucleotide primers. The use of such short primers may be enhanced by including gene 2.5 and 4 protein of T7 in the sequencing reaction.

DNA polymerases of this invention, (i.e., having the above properties) include modified T7-type polymerases. That is the DNA polymerase requires host thioredoxin as a sub-unit, and they are substantially identical to a modified T7 DNA polymerase or to equivalent enzymes isolated from related phage, such as T3, ΦI, ΦII, H, W31, gh-1, Y, A1122 and SP6. Each of these enzymes can be modified to have properties similar to those of the modified T7 enzyme. It is possible to isolate the enzyme from phage infected cells directly, but preferably the enzyme is isolated from cells which overproduce it. By substantially identical

is meant that the enzyme may have amino acid substitutions which do not affect the overall properties of the enzyme. One example of a particularly desirable amino acid substitution is one in which the natural enzyme is modified to remove any exonuclease activity. This modification may be performed at the genetic or chemical level (see below).

Cloning T7 polymerase

As an example of the invention we shall describe the cloning, overproduction, purification, modification and use of T7 DNA polymerase. This processive enzyme consists of two polypeptides tightly complexed in a one to one stoichiometry. One is the phage T7-encoded gene 5 protein of 84,000 daltons (Modrich et al. 150 J. Biol. Chem. 5515, 1975), the other is the *E. coli* encoded thioredoxin, of 12,000 daltons (Tabor et al., J. Biol, Chem. 262:16, 216, 1987). The thioredoxin is an accessory protein and attaches the gene 5 protein (the non-processive actual DNA polymerase) to the primer template. The natural DNA polymerase has a very active 3' to 5' exonuclease associated with it. This activity makes the polymerase useless for DNA sequencing and must be inactivated or modified before the polymerase can be used. This is readily performed, as described below, either chemically, by local oxidation of the exonuclease domain, or genetically, by modifying the coding region of the polymerase gene encoding this activity.

pTrx-2

In order to clone the trxA (thioredoxin) gene of *E. coli* wild type *E. coli* DNA was partially cleaved with Sau3A and the fragments ligated to BamHI-cleaved T7 DNA isolated from strain T7 ST9 (Tabor et al., in Thioredoxin and Glutaredoxin Systems: Structure and

Function (Holmgren et al., eds) pp. 285-300, Raven Press, NY; and Tabor et al., supra). The ligated DNA was transfected into E. coli trxA⁻ cells, the mixture plated onto trxA⁻ cells, and the resulting T7 plaques picked. Since T7 cannot grow without an active E. coli trxA gene only those phages containing the trxA gene could form plaques. The cloned trxA genes were located on a 470 base pair HincII fragment.

In order to overproduce thioreodoxin a plasmid, pTrx-2, was as constructed. Briefly, the 470 base pair HincII fragment containing the trxA gene was isolated by standard procedure (Maniatis et al., Cloning: A Laboratory Manual, Cold Spring Harbor Labs., Cold Spring Harbor, N.Y.), and ligated to a derivative of pBR322 containing a Ptac promoter (ptac-12, Amann et al., 25 Gene 167, 1983). Referring to Fig. 2, ptac-12, containing ß-lactamase and Col El origin, was cut with PvuII, to yield a fragment of 2290 bp, which was then ligated to two tandem copies of trxA (HincII fragment) using commercially available linkers (SmaI-BamHI polylinker), to form pTrx-2. The complete nucleotide sequence of pTrx-2 is shown in Figure 7. Thioredoxin production is now under the control of the tac promoter, and thus can be specifically induced, e.g. by IPTG (isopropyl ß-D-thiogalactoside).

pGP5-5 and mGP1-2

Some gene products of T7 are lethal when expressed in E. coli. An expression system was developed to facilitate cloning and expression of, lethal genes, based on the inducible expression of T7 RNA polymerase. Gene 5 protein is lethal in some E. coli strains and an example of such a system is described by Tabor et al. 82 Proc. Nat. Acad. Sci. 1074

(1985) where T7 gene 5 was placed under the control of the Φ10 promoter, and is only expressed when T7 RNA polymerase is present in the cell.

Briefly, pGP5-5 (Fig. 3) was constructed by standard procedures using synthetic BamHI linkers to join T7 fragment from 14306 (NdeI) to 16869 (AhaIII), containing gene 5, to the 560 bp fragment of T7 from 5667 (HincII) to 6166 (Fnu4H1) containing both the Φ1.1A and Φ1.1B promoters, which are recognized by T7 RNA polymerase, and the 3kb BamHI-HincII fragment of pACYC177 (Chang et al., 134 J. Bacteriol. 1141, 1978). The nucleotide sequence of the T7 inserts and linkers in shown in Fig. 8. In this plasmid gene 5 is only expressed when T7 RNA polymerase is provided in the cell.

Referring to Fig. 3, T7 RNA polymerase is provided on phage vector mGP1-2. This is similar to pGP1-2 (Tabor et al., *id.*) except that the fragment of T7 from 3133 (HaeIII) to 5840 (HinfI), containing T7 RNA polymerase was ligated, using linkers (BglII and SalI respectively), to BamHI-SalI cut M13 mp8, placing the polymerase gene under control of the *lac* promoter. The complete nucleotide sequence of mGP1-2 is shown in Fig. 9.

Since pGP5-5 and pTrx-2 have different origins of replication (respectively a P15A and a ColE1 origin) they can be tranformed into one cell simultaneously. pTrx-2 expresses large quantities of thioredoxin in the presence of IPTG. mGP1-2 can coexist in the same cell as these two plasmids and be used to regulate expression of T7-DNA polymerase from pGP5-5, simply by causing production of T7-RNA polymerase by inducing the *lac* promoter with, e.g., IPTG.

Overproduction of T7 DNA polymerase

There are several potential strategies for overproducing and reconstituting the two gene products of trxA and gene 5. The same cell strains and plasmids can be utilized for all the strategies. In the preferred strategy the two genes are co-overexpressed in the same cell. (This is because gene 5 is susceptible to proteases until thioredoxin is bound to it.) As described in detail below, one procedure is to place the two genes separately on each of two compatible plasmids in the same cell. Alternatively, the two genes could be placed in tandem on the same plasmid. It is important that the T7-gene 5 is placed under the control of a non-leaky inducible promoter, such as Φ1.1A, Φ1.1B and Φ10 of T7, as the synthesis of even small quantities of the two polypeptides together is toxic in most *E. coli* cells. By non-leaky is meant that less than 500 molecules of the gene product are produced, per cell generation time, from the gene when the promoter, controlling the gene's expression, is not activated. Preferably the T7 RNA polymerase expression system is used although other expression systems which utilize inducible promoters could also be used. A leaky promoter, e.g., plac, allows more than 500 molecules of protein to be synthesized, even when not induced, thus cells containing lethal genes under the control of such a promoter grow poorly and are not suitable in this invention. It is of course possible to produce these products in cells where they are not lethal, for example, the plac promoter is suitable in such cells.

In a second strategy each gene can be cloned and overexpressed separately. Using this strategy, the cells containing the individually overproduced polypeptides are combined prior to preparing the

extracts, at which point the two polypeptides form an active T7 DNA polymerase.

Example 1: Production of T7 DNA polymerase

E. coli strain 71.18 (Messing et al., Proc. Nat. Acad. Sci. 74:3642, 1977) is used for preparing stocks of mGP1-2. 71.18 is stored in 50% glycerol at -80°C. and is streaked on a standard minimal media agar plate. A single colony is grown overnight in 25 ml standard M9 media at 37°C, and a single plaque of mGP1-2 is obtained by titering the stock using freshly prepared 71.18 cells. The plaque is used to inoculate 10 ml 2X LB (2% Bacto-Tryptone, 1% yeast extract, 0.5% NaCl, 8mM NaOH) containing JM103 grown to an A_{590}=0.5. This culture will provide the phage stock for preparing a large culture of mGP1-2. After 3-12 hours, the 10 ml culture is centrifuged, and the supernatant used to infect the large (2L) culture. For the large culture, 4 X 500 ml 2X LB is inoculated with 4 X 5 ml 71.18 cells grown in M9, and is shaken at 37°C. When the large culture of cells has grown to an A_{590}=1.0 (approximately three hours), they are inoculated with 10 ml of supernatant containing the starter lysate of mGP1-2. The infected cells are then grown overnight at 37°C. The next day, the cells are removed by centrifugation, and the supernatant is ready to use for induction of K38/pGP5-5/pTrx-2 (see below). The supernatant can be stored at 4°C for approximately six months, at a titer ~5 X 10^{11} ϕ/ml. At this titer, 1 L of phage will infect 12 liters of cells at an A_{590}=5 with a multiplicity of infection of 15. If the titer is low, the mGP1-2 phage can be concentrated from the supernatant by dissolving NaCl (60 gm/liter) and PEG-6000 (65 gm/liter) in the supernatant, allowing the

mixture to settle at 0°C for 1-72 hours, and then centrifuging (7000 rpm for 20 min). The precipitate, which contains the mGP1-2 phage, is resuspended in approximately 1/20th of the original volume of M9 media.

K38/pGP5-5/pTrx-2 is the E. coli strain (genotype HfrC (λ)) containing the two compatible plasmids pGP5-5 and pTrx-2. pGP5-5 plasmid has a P15A origin of replication and expresses the kanamycin (Km) resistance gene. pTrx-2 has a ColEI origin of replication and expresses the ampicillin (Ap) resistance gene. The plasmids are introduced into K38 by standard procedures, selecting Km^R and Ap^R respectively. The cells K38/pGP5-5/pTrx-2 are stored in 50% glycerol at -80°C. Prior to use they are streaked on a plate containing 50μg/ml ampicillin and kanamycin, grown at 37°C overnight, and a single colony grown in 10 ml LB media containing 50μg/ml ampicillin and kanamycin, at 37°C for 4-6 hours. The 10 ml cell culture is used to inoculate 500 ml of LB media containing 50μg/ml ampicillin and kanamycin and shaken at 37°C overnight. The following day, the 500 ml culture is used to inoculate 12 liters of 2X LB-KPO_4 media (2% Bacto-Tryptone, 1% yeast extract, 0.5% NaCl, 20 mM KPO_4, 0.2% dextrose, and 0.2% casamino acids, pH 7.4), and grown with aeration in a fermentor at 37°C. When the cells reach an A_{590}=5.0 (i.e. logarithmic or stationary phase cells), they are infected with mGP1-2 at a multiplicity of infection of 10, and IPTG is added (final concentration 0.5mM). The IPTG induces production of thioredoxin and the T7 RNA polymerase in mGP1-2, and thence induces production of the cloned DNA

polymerase. The cells are grown for an additional 2.5 hours with stirring and aeration, and then harvested. The cell pellet is resuspended in 1.5 L 10% sucrose/20 mM Tris-HCl, pH 8.0/25 mM EDTA and re-spun. Finally, the cell pellet is resuspended in 200 ml 10% sucrose/20 mM Tris-HCl, pH 8/1.0 mM EDTA, and frozen in liquid N_2. From 12 liters of induced cells 70 gm of cell paste are obtained containing approximately 700 mg gene 5 protein and 100 mg thioredoxin.

K38/pTrx-2 (K38 containing pTrx-2 alone) overproduces thioredoxin, and it is added as a "booster" to extracts of K38/pGP5-5/pTrx-2 to insure that thioredoxin is in excess over gene 5 protein at the outset of the purification. The K38/pTrx-2 cells are stored in 50% glycerol at -80°C. Prior to use they are streaked on a plate containing 50 µg/ml ampicillin, grown at 37°C for 24 hours, and a single colony grown at 37°C overnight in 25 ml LB media containing 50 µg/ml ampicillin. The 25 ml culture is used to inoculate 2 L of 2X LB media and shaken at 37°C. When the cells reach an A_{590}=3.0, the ptac promoter, and thus thioredoxin production, is induced by the addition of IPTG (final concentration 0.5 mM). The cells are grown with shaking for an additional 12-16 hours at 37°C, harvested, resuspended in 600 ml 10% sucrose/20 mM Tris-HCl, pH 8.0/25 mM EDTA, and re-spun. Finally, the cells are resuspended in 40 ml 10% sucrose/20 mM Tris-HCl, pH 8/0.5 mM EDTA, and frozen in liquid N_2. From 2L of cells 16 gm of cell paste are obtained containing 150 mg of thioredoxin.

Assays for the polymerase involve the use of single-stranded calf thymus DNA (6mM) as a substrate. This is prepared immediately prior to use by

denaturation of double-stranded calf thymus DNA with 50 mM NaOH at 20°C for 15 min., followed by neutralization with HCl. Any purified DNA can be used as a template for the polymerase assay, although preferably it will have a length greater than 1,000 bases.

The standard T7 DNA polymerase assay used is a modification of the procedure described by Grippo et al. (246 J. Biol. Chem. 6867, 1971). The standard reaction mix (200 µl final volume) contains 40 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 100 nmol alkali-denatured calf thymus DNA, 0.3 mM dGTP, dATP, dCTP and [^{3}H]dTTP (20 cpm/pm), 50 µg/ml BSA, and varying amounts of T7 DNA polymerase. Incubation is at 37°C (10°C-45°C) for 30 min (5 min-60 min). The reaction is stopped by the addition of 3 ml of cold (0°C) 1 N HCl-0.1 M pyrophosphate. Acid-insoluble radioactivity is determined by the procedure of Hinkle et al. (250 J. Biol. Chem. 5523, 1974). The DNA is precipitated on ice for 15 min (5 min-12 hr), then precipitated onto glass-fiber filters by filtration. The filters are washed five times with 4 ml of cold (0°C) 0.1M HCl-0.1M pyrophosphate, and twice with cold (0°C) 90% ethanol. After drying, the radioactivity on the filters is counted using a non-aqueous scintillation fluor.

One unit of polymerase activity catalyzes the incorporation of 10 nmol of total nucleotide into an acid-soluble form in 30 min at 37°C, under the conditions given above. Native T7 DNA polymerase and modified T7 DNA polymerase (see below) have the same specific polymerase activity ± 20%, which ranges between 5,000-20,000 units/mg for native and 5,000-50,000 units/mg for modified polymerase) depending upon the preparation, using the standard assay conditions stated above.

T7 DNA polymerase is purified from the above extracts by precipitation and chromatography techniques. An example of such a purification follows.

An extract of frozen cells (200 ml K38/pGP5-5/pTrx-2 and 40 ml K38/pTrx-2) are thawed at 0°C overnight. The cells are combined, and 5 ml of lysozyme (15 mg/ml) and 10 ml of NaCl (5M) are added. After 45 min at 0°C, the cells are placed in a 37°C water bath until their temperature reaches 20°C. The cells are then frozen in liquid N_2. An additional 50 ml of NaCl (5M) is added, and the cells are thawed in a 37°C water bath. After thawing, the cells are gently mixed at 0°C for 60 min. The lysate is centrifuged for one hr at 35,000 rpm in a Beckman 45Ti rotor. The supernatant (250 ml) is fraction I. It contains approximately 700 mg gene 5 protein and 250 mg of thioredoxin (a 2:1 ratio thioredoxin to gene 5 protein).

90 gm of ammonium sulphate is dissolved in fraction I (250 ml) and stirred for 60 min. The suspension is allowed to sit for 60 min, and the resulting precipitate collected by centrifugation at 8000 rpm for 60 min. The precipitate is redissolved in 300 ml of 20 mM Tris-HCl pH 7.5/5 mM 2-mercaptoethanol/0.1 mM EDTA/10% glycerol (Buffer A). This is fraction II.

A column of Whatman DE52 DEAE (12.6 cm^2 x 18 cm) is prepared and washed with Buffer A. Fraction II is dialyzed overnight against two changes of 1 L of Buffer A each until the conductivity of Fraction II has a conductivity equal to that of Buffer A containing 100 mM NaCl. Dialyzed Fraction II is applied to the column at a flow rate of 100 ml/hr, and washed with 400 ml of Buffer A containing 100 mM NaCl. Proteins are eluted

with a 3.5 L gradient from 100 to 400 mM NaCl in Buffer A at a flow rate of 60 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 200 mM NaCl, are pooled. This is fraction III (190 ml).

A column of Whatman P11 phosphocellulose (12.6 cm^2 x 12 cm) is prepared and washed with 20 mM KPO_4 pH 7.4/5 mM 2-mercaptoethanol/0.1 mM EDTA/10 % glycerol (Buffer B). Fraction III is diluted 2-fold (380 ml) with Buffer B, then applied to the column at a flow rate of 60 ml/hr, and washed with 200 ml of Buffer B containing 100mM KCl. Proteins are eluted with a 1.8 L gradient from 100 to 400 mM KCl in Buffer B at a flow rate of 60 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 300 mM KCl, are pooled. This is fraction IV (370 ml).

A column of DEAE-Sephadex A-50 (4.9 cm^2 x 15 cm) is prepared and washed with 20 mM Tris-HCl 7.0/0.1 mM dithiothreitol/0.1 mM EDTA/10% glycerol (Buffer C). Fraction IV is dialyzed against two changes of 1 L Buffer C to a final conductivity equal to that of Buffer C containing 100 mM NaCl. Dialyzed fraction IV is applied to the column at a flow rate of 40 ml/hr, and washed with 150 ml of Buffer C containing 100 mM NaCl. Proteins are eluted with a 1 L gradient from 100 to 300 mM NaCl in Buffer C at a flow rate of 40 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 210 mM NaCl, are pooled. This is fraction V (120 ml).

A column of BioRad HTP hydroxylapatite (4.9 cm^2 x 15 cm) is prepared and washed with 20 mM KPO_4, pH 7.4/10 mM 2-mercaptoethanol/2 mM Na citrate/10% glycerol (Buffer D). Fraction V is dialyzed against two changes of 500 ml Buffer D each. Dialyzed fraction V is applied to the column at a flow rate of 30 ml/hr, and

washed with 100 ml of Buffer D. Proteins are eluted with a 900 ml gradient from 0 to 180 mM KPO_4, pH 7.4 in Buffer D at a flow rate of 30 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 50 mM KPO_4, are pooled. This is fraction VI (130 ml). It contains 270 mg of homogeneous T7 DNA polymerase.

Fraction VI is dialyzed versus 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/0.1 mM EDTA/50% glycerol. This is concentrated fraction VI (~65 ml, 4 mg/ml), and is stored at -20°C.

The isolated T7 polymerase has exonuclease activity associated with it. As stated above this must be inactivated. An example of inactivation by chemical modification follows.

Concentrated fraction VI is dialyzed overnight against 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/10% glycerol to remove the EDTA present in the storage buffer. After dialysis, the concentration is adjusted to 2 mg/ml with 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/10% glycerol, and 30 ml (2mg/ml) aliquots are placed in 50 ml polypropylene tubes. (At 2 mg/ml, the molar concentration of T7 DNA polymerase is 22 μM.)

Dithiothreitol (DTT) and ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 6H_2O$) are prepared fresh immediately before use, and added to a 30 ml aliquot of T7 DNA polymerase, to concentrations of 5 mM DTT (0.6 ml of a 250 mM stock) and 20μM $Fe(NH_4)_2(SO_4)_2 6H_2O$ (0.6 ml of a 1 mM stock). During modification the molar concentrations of T7 DNA polymerase and iron are each approximately 20 μM, while DTT is in 250X molar excess.

The modification is carried out at 0°C under a saturated oxygen atmosphere as follows. The reaction mixture is placed on ice within a dessicator, the dessicator is purged of air by evacuation and subsequently filled with 100% oxygen. This cycle is repeated three times. The reaction can be performed in air (20% oxygen), but occurs at one third the rate.

The time course of loss of exonuclease activity is shown in Fig. 4. ^{3}H-labeled double-stranded DNA (6 cpm/pmol) was prepared from bacteriophage T7 as described by Richardson (15 J. Molec. Biol. 49, 1966). ^{3}H-labeled single-stranded T7 DNA was prepared immediately prior to use by denaturation of double-stranded ^{3}H-labeled T7 DNA with 50 mM NaOH at 20°C for 15 min, followed by neutralization with HCl. The standard exonuclease assay used is a modification of the procedure described by Chase et al. (<u>supra</u>). The standard reaction mixture (100 µl final volume) contained 40 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 60 nmol ^{3}H-labeled single-stranded T7 DNA (6 cpm/pm), and varying amounts of T7 DNA polymerase. ^{3}H-labeled double-stranded T7 DNA can also be used as a substrate. Also, any uniformly radioactively labeled DNA, single- or double-stranded, can be used for the assay. Also, 3' end labeled single- or double-stranded DNA can be used for the assay. After incubation at 37°C for 15 min, the reaction is stopped by the addition of 30 µl of BSA (10mg/ml) and 25 µl of TCA (100% w/v). The assay can be run at 10°C-45°C for 1-60 min. The DNA is precipitated on ice for 15 min (1 min - 12 hr), then centrifuged at 12,000 g for 30 min (5 min - 3 hr). 100 µl of the supernatant is used to determine the acid-soluble radioactivity by adding it to

400 µl water and 5 ml of aqueous scintillation cocktail.

One unit of exonuclease activity catalyzes the acid solubilization of 10 nmol of total nucleotide in 30 min under the conditions of the assay. Native T7 DNA polymerase has a specific exonuclease activity of 5000 units/mg, using the standard assay conditions stated above. The specific exonuclease activity of the modified T7 DNA polymerase depends upon the extent of chemical modification, but ideally is at least 10-100-fold lower than that of native T7 DNA polymerase, or 500 to 50 or less units/mg using the standard assay conditions stated above. When double stranded substrate is used the exonuclease activity is about 7-fold higher.

Under the conditions outlined, the exonuclease activity decays exponentially, with a half-life of decay of eight hours. Once per day the reaction vessel is mixed to distribute the soluble oxygen, otherwise the reaction will proceed more rapidly at the surface where the concentration of oxygen is higher. Once per day 2.5 mM DTT (0.3 ml of a fresh 250 mM stock to a 30 ml reaction) is added to replenish the oxidized DTT.

After eight hours, the exonuclease activity of T7 DNA polymerase has been reduced 50%, with negligible loss of polymerase activity. The 50% loss may be the result of the complete inactivation of exonuclease activity of half the polymerase molecules, rather than a general reduction of the rate of exonuclease activity in all the molecules. Thus, after an eight hour reaction all the molecules have normal polymerase activity, half the molecules have normal exonuclease activity, while the other half have <0.1% of their original exonuclease activity.

When 50% of the molecules are modified (an eight hour reaction), the enzyme is suitable, although suboptimal, for DNA sequencing. For more optimum quality of DNA sequencing, the reaction is allowed to proceed to greater than 99% modification (having less than 50 units of exonuclease activity), which requires four days.

After four days, the reaction mixture is dialyzed against 2 changes of 250 ml of 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/0.1 mM EDTA/50% glycerol to remove the iron. The modified T7 DNA polymerase (~4 mg/ml) is stored at -20°C.

The reaction mechanism for chemical modification of T7 DNA polymerase depends upon reactive oxygen species generated by the presence of reduced transition metals such as Fe^{2+} and oxygen. A possible reaction mechanism for the generation of hydroxyl radicals is outlined below:

(1) $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot}$
(2) $2\ O_2^{\cdot} + 2\ H^+ \rightarrow H_2O_2 + O_2$
(3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$

In equation 1, oxidation of the reduced metal ion yields superoxide radical, $O_2^{\cdot}$. The superoxide radical can undergo a dismutation reaction, producing hydrogen peroxide (equation 2). Finally, hydrogen peroxide can react with reduced metal ions to form hydroxyl radicals, $OH^{\cdot}$ (the Fenton reaction, equation

3). The oxidized metal ion is recycled to the reduced form by reducing agents such as dithiothreitol (DTT).

These reactive oxygen species probably inactivate proteins by irreversibly chemically altering specific amino acid residues. Such damage is observed in SDS-PAGE of fragments of gene 5 produced by CNBr or trypsin. Some fragments disappear, high molecular weight cross linking occurs, and some fragments are broken into two smaller fragments.

As previously mentioned, oxygen, a reducing agent (e.g. DTT, 2-mercaptoethanol) and a transition metal (e.g. iron) are essential elements of the modification reaction. The reaction occurs in air, but is stimulated three-fold by use of 100% oxygen. The reaction will occur slowly in the absence of added transition metals due to the presence of trace quantities of transition metals (1-2μM) in most buffer preparations.

As expected, inhibitors of the modification reaction include anaerobic conditions (e.g., N_2) and metal chelators (e.g. EDTA, citrate, nitrilotriacetate). In addition, the enzymes catalase and superoxide dismutase may inhibit the reaction, consistent with the essential role of reactive oxygen species in the generation of modified T7 DNA polymerase.

As an alternative procedure, it is possible to genetically mutate the T7 gene 5 to specifically inactivate the exonuclease domain of the protein. The T7 gene 5 protein purified from such mutants is ideal for use in DNA sequencing without the need to chemically inactivate the exonuclease by oxidation and without the secondary damage that inevitably occurs to the protein during chemical modification.

Genetically modified T7 DNA polymerase can be isolated by randomly mutagenizing the gene 5 and then

screening for those mutants that have lost exonuclease activity, without loss of polymerase activity. Mutagenesis is performed as follows. Single-stranded DNA containing gene 5 (e.g., cloned in pEMBL-8, a plasmid containing an origin for single stranded DNA replication) under the control of a T7 RNA polymerase promoter is prepared by standard procedure, and treated with two different chemical mutagens: hydrazine, which will mutate C's and T's, and formic acid, which will mutate G's and A's. Myers et al. 229 Science 242, 1985. The DNA is mutagenized at a dose which results in an average of one base being altered per plasmid molecule. The single-stranded mutagenized plasmids are then primed with a universal 17-mer primer (see above), and used as templates to synthesize the opposite strands. The synthesized strands contain randomly incorporated bases at positions corresponding to the mutated bases in the templates. The double-stranded mutagenized DNA is then used to transform the strain K38/pGP1-2, which is strain K38 containing the plasmid pGP1-2 (Tabor et al., <u>supra</u>). Upon heat induction this strain expresses T7 RNA polymerase. The transformed cells are plated at 30°C, with approximately 200 colonies per plate.

Screening for cells having T7 DNA polymerase lacking exonuclease activity is based upon the following finding. The 3' to 5' exonuclease of DNA polymerases serves a proofreading function. When bases are misincorporated, the exonuclease will remove the newly incorporated base which is recognized as "abnormal". This is the case for the analog of dATP, etheno-dATP, which is readily incorporated by T7 DNA polymerase in place of dATP. However, in the presence of the 3' to 5' exonuclease of T7 DNA polymerase, it is excised as

rapidly as it is incorporated, resulting in no net DNA synthesis. As shown in figure 6, using the alternating copolymer poly d(AT) as a template, native T7 DNA polymerase catalyzes extensive DNA synthesis only in the presence of dATP, and not etheno-dATP. In contrast, modified T7 DNA polymerase, because of its lack of an associated exonuclease, stably incorporates etheno-dATP into DNA at a rate comparable to dATP. Thus, using poly d(AT) as a template, and dTTP and etheno-dATP as precursors, native T7 DNA polymerase is unable to synthesize DNA from this template, while T7 DNA polymerase which has lost its exonuclease activity will be able to use this template to synthesize DNA.

The procedure for lysing and screening large number of colonies is described in Raetz (72 Proc. Nat. Acad. Sci. 2274, 1975). Briefly, the K38/pGP1-2 cells transformed with the mutagenized gene 5-containing plasmids are transferred from the petri dish, where they are present at approximately 200 colonies per plate, to a piece of filter paper ("replica plating"). The filter paper discs are then placed at 42°C for 60 min to induce the T7 RNA polymerase, which in turn expresses the gene 5 protein. Thioredoxin is constitutively produced from the chromosomal gene. Lysozyme is added to the filter paper to lyse the cells. After a freeze thaw step to ensure cell lysis, the filter paper discs are incubated with poly d(AT), [α^{32}P]dTTP and etheno-dATP at 37°C for 60 min. The filter paper discs are then washed with acid to remove the unincorporated [^{32}P]dATP. DNA will precipitate on the filter paper in acid, while nucleotides will be soluble. The washed filter paper is then used to expose X-ray film. Colonies which have induced an active T7 DNA polymerase which is deficient

in its exonuclease will have incorporated acid-insoluble ^{32}P, and will be visible by autoradiography. Colonies expressing native T7 DNA polymerase, or expressing a T7 DNA polymerase defective in polymerase activity, will not appear on the autoradiograph.

Colonies which appear positive are recovered from the master petri dish containing the original colonies. Cells containing each potential positive clone will be induced on a larger scale (one liter) and T7 DNA polymerase purified from each preparation to ascertain the levels of exonuclease associated with each mutant. Those low in exonuclease are appropriate for DNA sequencing.

Directed mutagenesis may also be used to isolate genetic mutants in the exonuclease domain of the T7 gene 5 protein. The following is an example of this procedure.

T7 DNA polymerase with reduced exonuclease activity (modified T7 DNA polymerase) can also be distinguished from native T7 DNA polymerase by its ability to synthesize through regions of secondary structure. Thus, with modified DNA polymerase, DNA synthesis from a labeled primer on a template having secondary structure will result in significantly longer extensions, compared to unmodified or native DNA polymerase. This assay provides a basis for screening for the conversion of small percentages of DNA polymerase molecules to a modified form.

The above assay was used to screen for altered T7 DNA polymerase after treatment with a number of chemical reagents. Three reactions resulted in conversion of the enzyme to a modified form. The first is treatment with iron and a reducing agent, as

described above. The other two involve treatment of the enzyme with photooxidizing dyes, Rose Bengal and methylene blue, in the presence of light. The dyes must be titrated carefully, and even under optimum conditions the specificity of inactivation of exonuclease activity over polymerase activity is low, compared to the high specificity of the iron-induced oxidation. Since these dyes are quite specific for modification of histidine residues, this result strongly implicates histidine residues as an essential species in the exonuclease active site.

There are 23 histidine residues in T7 gene 5 protein. Eight of these residues lie in the amino half of the protein, in the region where, based on the homology with the large fragment of E. coli DNA polymerase I, the exonuclease domain may be located (Ollis et al. Nature 313, 818. 1984). As described below, seven of the eight histidine residues were mutated individually by synthesis of appropriate oligonucleotides, which were then incorporated into gene 5. These correspond to mutants 1, and 6-10 in table 1.

The mutations were constructed by first cloning the T7 gene 5 from pGP5-3 (Tabor et al., J. Biol. Chem. 282, 1987) into the SmaI and HindIII sites of the vector M13 mp18, to give mGP5-2. (The vector used and the source of gene 5 are not critical in this procedure.) Single-stranded mGP5-2 DNA was prepared from a strain that incorporates deoxyuracil in place of deoxythymidine (Kunkel, Proc. Natl. Acad. Sci. USA 82, 488, 1985). This procedure provides a strong selection for survival of only the synthesized strand (that containing the mutation) when transfected into wild-type E.coli, since the strand containing uracil will be preferentially degraded.

Mutant oligonucleotides, 15-20 bases in length, were synthesized by standard procedures. Each oligonucleotide was annealed to the template, extended using native T7 DNA polymerase, and ligated using T4 DNA ligase. Covalently closed circular molecules were isolated by agarose gel electrophoresis, run in the presence of 0.5µg/ml ethidium bromide. The resulting purified molecules were then used to transform E. coli 71.18. DNA from the resulting plaques was isolated and the relevant region sequenced to confirm each mutation.

The following summarizes the oligonucleotides used to generate genetic mutants in the gene 5 exonuclease. The mutations created are underlined. Amino acid and base pair numbers are taken from Dunn et al., 166 J. Molec. Biol. 477, 1983. The relevant wild type sequences of the region of gene 5 mutated are also shown.

Wild type sequence:

```
109 (aa)                                                        122 123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GCT TTG GAG
14677 (T7 bp)
```

Mutation 1: His 123 → Ser 123

Primer used: 5' CGC TTT GGA TCC TCC GCT TTG 3'

Mutant sequence:

```
                                                                123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser Ser Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGA TCC TCC GCT TTG GAG
```

Mutation 2: Deletion of Ser 122 and His 123

Primer used: 5' GGA AAA CGC TTT GGC GCC TTG GAG GCG 3'

```
                              Δ
                        6 base deletion
```

Mutant sequence:

```
                                                     122 123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly ··· ··· Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGC --- --- GCC TTG GAG
```

Mutation 3: Ser 122, His 123 → Ala 122, Glu 123

Primer used: 5' CGC TTT GGG GCT GAG GCT TTG G 3'

Mutant sequence:

```
                                                             122 123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ala Glu Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG GCT GAG GCT TTG GAG
```

Mutation 4: Lys 118, Arg 119 → Glu 118, Glu 119

Primer used: 5' 5' G CCC GGG GAA GAG TTT GGG TCT CAC GC 3'

Mutant sequence:

```
                                     118 119
Leu Leu Arg Ser Gly Lys Leu Pro Gly Glu Glu Phe Gly Ser His Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGG GAA GAG TTT GGG TCT CAC GCT TTG GAG
```

Mutation 5:. Arg 111, Ser 112, Lys 114 → Glu 111, Ala 112, Glu 114

Primer used : 5' G GGT CTT CTG GAA GCC GGC GAG TTG CCC GG 3'

Mutant sequence:

```
        111 112     114
Leu Leu Glu Ala Gly Glu Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu
Glu
CTT CTG GAA GCC GGC GAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GCT TTG GAG
```

Mutation 6: His 59, His 62 → Ser 59, Ser 62

Primer used: 5' ATT GTG TTC TCC AAC GGA TCC AAG TAT GAC G 3'

Wild-type sequence:

```
      aa: 55              59          62
          Leu Ile Val Phe His Asn Gly His Lys Tyr Asp Val
          CTT ATT GTG TTC CAC AAC GGT CAC AAG TAT GAC GTT
   T7 bp: 14515
```

Mutant sequence:

```
                          59          62
          Leu Ile Val Phe Ser Asn Gly Ser Lys Tyr Asp Val
          CTT ATT GTG TTC TCC AAC GGA TCC AAG TAT GAC GTT
```

Mutation 7: His 82 → Ser 82

Primer used: 5' GAG TTC TCC CTT CCT CG 3'

Wild-type sequence:

```
     aa: 77                    82
         Leu Asn Arg Glu Phe His Leu Pro Arg Glu Asn
         TTG AAC CGA GAG TTC CAC CTT CCT CGT GAG AAC
 T7 bp:  14581
```

Mutant sequence:

```
                               82
         Leu Asn Arg Glu Phe Ser Leu Pro Arg Glu Asn
         TTG AAC CGA GAG TTC TCC CTT CCT CGT GAG AAC
```

Mutation 8: Arg 96, His 99 → Leu 96, Ser 99

Primer used: 5' CTG TTG ATT TCT TCC AAC CTC 3'

Wild-type sequence:

```
     aa: 93          96          99
         Val Leu Ser Arg Leu Ile His Ser Asn Leu Lys Asp Thr Asp
         GTG TTG TCA CGT TTG ATT CAT TCC AAC CTC AAG GAC ACC GAT
 T7 bp:  14629
```

Mutant sequence:

```
                     96          99
         Val Leu Ser Leu Leu Ile Ser Ser Asn Leu Lys Asp Thr Asp
         GTG TTG TCA CTG TTG ATT TCT TCC AAC CTC AAG GAC ACC GAT
```

Mutation 9: His 190 → Ser 190

Primer used: 5' CT GAC AAA TCT TAC TTC CCT 3'

Wild-type sequence:

```
     aa: 185                 190
         Leu Leu Ser Asp Lys His Tyr Phe Pro Pro Glu
         CTA CTC TCT GAC AAA CAT TAC TTC CCT CCT GAG
 T7 bp:  14905
```

Mutant sequence:

```
                             190
         Leu Leu Ser Asp Lys Ser Tyr Phe Pro Pro Glu
         CTA CTC TCT GAC AAA TCT TAC TTC CCT CCT GAG
```

Mutation 10: His 218 → Ser 218

Primer used: 5' GAC ATT GAA TCT CGT GCT GC 3'

Wild-type sequence:

```
    aa: 214             218
        Val Asp Ile Glu His Arg Ala Ala Trp Leu Leu
        GTT GAC ATT GAA CAT CGT GCT GCA TGG CTG CTC
T7 bp:  14992
```

Mutant sequence:

```
                        218
        Val Asp Ile Glu Ser Arg Ala Ala Trp Leu Leu
        GTT GAC ATT GAA TCT CGT GCT GCA TGG CTG CTC
```

Mutation 11: Deletion of amino acids 118 to 123

```
Primer used:  5' C GGC AAG TTG CCC GGG GCT TTG GAG GCG TGG G 3'
                                     Δ
                             18 base deletion
```

Wild-type sequence:

```
109 (aa)                                118                 122 123             126
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GCT TTG GAG
14677 (T7 bp)
```

Mutant sequence:

```
                                117                          124
Leu Leu Arg Ser Gly Lys Leu Pro Gly·····(6 amino acids)······Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGG········(18 bases)········GCT TTG GAG
```

Mutation 12: His 123 → Glu 123

Primer used: 5' GGG TCT GAG GCT TTG G 3'

Mutant sequence:

```
                                                            123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser Glu Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT GAG GCT TTG GAG
```

Mutation 13: (Arg 131, Lys 136, Lys 140, Lys 144, Arg 145 →
Glu 131, Glu 136, Glu 140, Glu 144, Glu 145)

Primer used: 5' GGT TAT GAG CTC GGC GAG ATG GAG GGT GAA TAC GAA GAC GAC TTT GAG GAA
CTT GAA G 3'

Wild-type sequence:

```
129(aa) 131                     136              140              144 145
Gly Tyr Arg Leu Gly Glu Met Lys Gly Glu Tyr Lys Asp Asp Phe Lys Arg Met Leu Glu Glu
GGT TAT CGC TTA GGC GAG ATG AAG GGT GAA TAC AAA GAC GAC TTT AAG CGT ATG CTT GAA G
14737 (T7 bp)
```

Mutant sequence:

```
129(aa) 131                     136              140              144 145
Gly Tyr Glu Leu Gly Glu Met Glu Gly Glu Tyr Glu Asp Asp Phe Glu Glu Met Leu Glu Glu
GGT TAT GAG CTC GGC GAG ATG GAG GGT GAA TAC GAA GAC GAC TTT GAG GAA ATG CTT GAA G
14737 (T7 bp)
```

Each mutant gene 5 protein was produced by infection of the mutant phage into K38/pGP1-2, as follows. The cells were grown at 30°C to an A_{590}=1.0. The temperature was shifted to 42°C for 30 min., to induce T7 RNA polymerase. IPTG was added to 0.5 mM, and a lysate of each phage was added at a moi=10. Infected cells were grown at 37°C for 90 min. The cells were then harvested and extracts prepared by standard procedures for T7 gene 5 protein.

Extracts were partially purified by passage over a phosphocellulose and DEAE A-50 column, and assayed by measuring the polymerase and exonuclease activities directly, as described above. The results are shown in Table 1.

Table 1
SUMMARY OF EXONUCLEASE AND POLYMERASE ACTIVITIES OF T7 GENE 5 MUTANTS

Mutant	Exonuclease activity, %	Polymerase activity, %
[Wild-type]	[100][a]	[100][b]
Mutant 1 (His 123 → Ser 123)	10-25	>90
Mutant 2 (Δ Ser 122, His 123)	0.2-0.4	>90
Mutant 3 (Ser 122, His 123 → Ala 122, Glu 123)	<2	>90

Table 1
SUMMARY OF EXONUCLEASE AND POLYMERASE ACTIVITIES OF T7 GENE 5 MUTANTS

Mutant	Exonuclease activity, %	Polymerase activity, %
Mutant 4 (Lys 118, Arg 119 → Glu 118, Glu 119)	<30	>90
Mutant 5 (Arg 111, Ser 112, Lys 114 → Glu 111, Ala 112, Glu 114)	>75	>90
Mutant 6 (His 59, His 62 → Ser 59, Ser 62)	>75	>90
Mutant 7 (His 82 → Ser 82)	>75	>90
Mutant 8 (Arg 96, His 99 → Leu 96, Ser 99)	>75	>90
Mutant 9 (His 190 → Ser 190)	>75	>90
Mutant 10 (His 218 → Ser 218)	>75	>90
Mutant 11 (Δ Lys 118, Arg 119, Phe 120, Gly 121, Ser 122, His 123)	<0.02	>90
Mutant 12 (His 123 → Glu 123)	<30	>90
Mutant 13 (Arg 131, Lys 136, Lys 140, Lys 144, Arg 145 → Glu 131, Glu 136, Glu 140, Glu 144, Glu 145)	<30	>90

a. Exonuclease activity was measured on single stranded [^{3}H]T7 DNA. 100% exonuclease activity corresponds to 5,000 units/mg.

b. Polymerase activity was measured using single-stranded calf thymus DNA. 100% polymerase activity corresponds to 8,000 units/mg.

Of the seven histidines tested, only one (His 123: mutant 1) has the enzymatic activities characteristic of modified T7 DNA polymerase. T7 gene 5 protein was purified from this mutant using DEAE-cellulose, phosphocellulose, DEAE-Sephadex and hydroxylapatite chromatography. While the polymerase activity was nearly normal (>90% the level of the native enzyme), the exonuclease activity was reduced 4 to 10-fold.

A variant of this mutant was constructed in which both His 123 and Ser 122 were deleted. The gene 5 protein purified from this mutant has a 200-500 fold lower exonuclease activity, again with retention of >90% of the polymerase activity.

These data strongly suggest that His 123 lies in the active site of the exonuclease domain of T7 gene 5 protein. Furthermore, it is likely that the His 123 is in fact the residue being modified by the oxidation involving iron, oxygen and a reducing agent, since such oxidation has been shown to modify histidine residues in other proteins (Levine, J. Biol. Chem. 258: 11823, 1983; and Hodgson et al. Biochemistry 14: 5294, 1975). The level of residual exonuclease in mutant 11 is comparable to the levels obtainable by chemical modification.

Although mutations at His residues are described, mutations at nearby sites or even at distant sites may also produce mutant enzymes suitable in this invention, e.g., lys and arg (mutants 4 and 15). Similarly, although mutations in some His residues have little effect on exonuclease activity that does not necessarily indicate that mutations near these residues will not affect exonuclease activity.

Mutations which are especially effective include those having deletions of 2 or more amino acids, preferably 6-8, for example, near the His-123 region. Other mutations should reduce exonuclease activity further, or completely.

As an example of the use of these mutant strains the following is illustrative. A pGP5-6 (mutation 11)-containing strain has been deposited with the ATCC (see below). The strain is grown as described above and induced as described in Taber et al. J. Biol. Chem. 262:16212 (1987). K38/pTrx-2 cells may be added to increase the yield of genetically modified T7 DNA polymerase.

The above noted deposited strain also contains plasmid pGP1-2 which expresses T7 RNA polymerase. This plasmid is described in Tabor et al., Proc. Nat. Acad. Sci. USA 82:1074, 1985 and was deposited with the ATCC on March 22, 1985 and assigned the number 40,175.

Referring to Fig. 10, pGP5-6 includes the following segments:

1. EcoRI-SacI-SmaI-BamHI polylinker sequence from M13 mp10 (21bp).

2. T7 bp 14309 to 16747, that contains the T7 gene 5, with the following modifications:

T7 bp 14703 is changed from an A to a G, creating a SmaI site.

T7 bp 14304 to 14321 inclusive are deleted (18 bp).

3. SalI-PstI-HindIII polylinker sequence from M13 mp 10 (15 bp)

4. pBR322 bp 29 (HindIII site) to pBR322 bp 375 (BamHI site).

5. T7 bp 22855 to T7 bp 22927, that contains the T7 RNA Polymerase promoter $\phi 10$, with BamHI linkers inserted at each end (82 bp).

6. pBR322 bp 375 (BamHI site) to pBR322 bp 4361 (EcoRI site).

DNA Sequencing Using Modified T7-type DNA Polymerase

DNA synthesis reactions using modified T7-type DNA polymerase result in chain-terminated fragments of uniform radioactive intensity, throughout the range of several bases to thousands of bases in length. There is virtually no background due to terminations at sites independent of chain terminating agent incorporation (i.e. at pause sites or secondary structure impediments).

Sequencing reactions using modified T7-type DNA polymerase consist of a pulse and chase. By pulse is meant that a short labelled DNA fragment is synthesized; by chase is meant that the short fragment is lengthened until a chain terminating agent is incorporated. The rationale for each step differs from conventional DNA sequencing reactions. In the pulse, the reaction is incubated at 0°C-37°C for 0.5-4 min in the presence of high levels of three nucleotide triphosphates (e.g., dGTP, dCTP and dTTP) and limiting levels of one other labelled, carrier-free, nucleotide triphosphate, e.g., [^{35}S] dATP. Under these conditions the modified polymerase is unable to exhibit its processive character, and a population of radioactive fragments will be synthesized ranging in size from a few bases to several hundred bases. The purpose of the pulse is to radioactively label each primer, incorporating maximal radioactivity while using minimal levels of radioactive

nucleotides. In this example, two conditions in the pulse reaction (low temperature, e.g., from 0-20°C, and limiting levels of dATP, e.g., from 0.1μM to 1μM) prevent the modified T7-type DNA polymerase from exhibiting its processive character. Other essential environmental components of the mixture will have similar effects, e.g., limiting more than one nucleotide triphosphate or increasing the ionic strength of the reaction. If the primer is already labelled (e.g., by kinasing) no pulse step is required.

In the chase, the reaction is incubated at 45°C for 1-30 min in the presence of high levels (50-500μM) of all four deoxynucleoside triphosphates and limiting levels (1-50μM) of any one of the four chain terminating agents, e.g., dideoxynucleoside triphosphates, such that DNA synthesis is terminated after an average of 50-600 bases. The purpose of the chase is to extend each radioactively labeled primer under conditions of processive DNA synthesis, terminating each extension exclusively at correct sites in four separate reactions using each of the four dideoxynucleoside triphosphates. Two conditions of the chase (high temperature, e.g., from 30-50°C) and high levels (above 50μM) of all four deoxynucleoside triphosphates) allow the modified T7-type DNA polymerase to exhibit its processive character for tens of thousands of bases; thus the same polymerase molecule will synthesize from the primer-template until a dideoxynucleotide is incorporated. At a chase temperature of 45°C synthesis occurs at >700 nucleotides/sec. Thus, for sequencing reactions the chase is complete in less than a second. <u>ssb</u> increases processivity, for example, when using dITP, or when using low temperatures or high ionic strength, or low levels of triphosphates throughout the sequencing reaction.

Either [α^{35}S]dATP,[α^{32}P]dATP or fluorescently labelled nucleotides can be used in the DNA sequencing reactions with modified T7-type DNA polymerase. If the fluorescent analog is at the 5' end of the primer, then no pulse step is required.

Two components determine the average extensions of the synthesis reactions. First is the length of time of the pulse reaction. Since the pulse is done in the absence of chain terminating agents, the longer the pulse reaction time, the longer the primer extensions. At 0°C the polymerase extensions average 10 nucleotides/sec. Second is the ratio of deoxyribonucleoside triphosphates to chain terminating agents in the chase reaction. A modified T7-type DNA polymerase does not discriminate against the incorporation of these analogs, thus the average length of extension in the chase is four times the ratio of the deoxynucleoside triphosphate concentration to the chain terminating agent concentration in the chase reaction. Thus, in order to shorten the average size of the extensions, the pulse time is shortened, e.g., to 30 sec. and/or the ratio of chain terminating agent to deoxynucleoside triphosphate concentration is raised in the chase reaction. This can be done either by raising the concentration of the chain terminating agent or lowering the concentration of deoxynucleoside triphosphate. To increase the average length of the extensions, the pulse time is increased, e.g., to 3-4 min. and/or the concentration of chain terminating agent is lowered (e.g., from 20μM to 2μM) in the chase reaction.

Example 2: DNA sequencing using modified T7 DNA polymerase

The following is an example of a sequencing protocol using dideoxy nucleotides as terminating agents.

9μl of single-stranded M13 DNA (mGP1-2, prepared by standard procedures) at 0.7 mM concentration is mixed with 1 μl of complementary sequencing primer (standard universal 17-mer, 0.5 pmole primer / μl) and 2.5 μl 5X annealing buffer (200 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$) heated to 65°C for 3 min, and slow cooled to room temperature over 30 min. In the pulse reaction, 12.5 μl of the above annealed mix was mixed with 1 μl dithiothreitol 0.1 M, 2 μl of 3 dNTPs (dGTP, dCTP, dTTP) 3 mM each (P.L Biochemicals, in TE), 2.5 μl [α^{35}S]dATP, (1500 Ci/mmol, New England Nuclear) and 1 μl of modified T7 DNA polymerase described in Example 1 (0.4 mg/ml, 2500 units/ml, i.e. 0.4 μg, 2.5 units) and incubated at 0°C, for 2 min, after vortexing and centrifuging in a microfuge for 1 sec. The time of incubation can vary from 30 sec to 20 min and temperature can vary from 0°C to 37°C. Longer times are used for determining sequences distant from the primer.

4.5 μl aliquots of the above pulse reaction are added to each of four tubes containing the chase mixes, preheated to 45°C. The four tubes, labeled G, A, T, C, each contain trace amounts of either dideoxy (dd) G, A, T, or C (P-L Biochemicals). The specific chase solutions are given below. Each tube contains 1.5 μl dATP 1mM, 0.5 μl 5X annealing buffer (200 mM Tris-HCl, pH 7.5, 50mM $MgCl_2$), and 1.0 μl ddNTP 100 μM (where ddNTP corresponds to ddG,A,T or C in the respective tubes). Each chase reaction is incubated at 45°C (or 30°C-50°C) for 10 min, and then 6 μl of stop solution (90% formamide, 10mM EDTA, 0.1% xylenecyanol) is added to each tube, and the tube placed on ice. The chase times can vary from 1-30 min.

The sequencing reactions are run on standard, 6% polyacrylamide sequencing gel in 7M urea, at 30 Watts for 6 hours. Prior to running on a gel the reactions are heated to 75°C for 2 min. The gel is fixed in 10% acetic acid, 10% methanol, dried on a gel dryer, and exposed to Kodak OM1 high-contrast autoradiography film overnight.

Example 3: DNA sequencing using limiting concentrations of dNTPs

In this example DNA sequence analysis of mGP1-2 DNA is performed using limiting levels of all four deoxyribonucleoside triphosphates in the pulse reaction. This method has a number of advantages over the protocol in example 2. First, the pulse reaction runs to completion, whereas in the previous protocol it was necessary to interrupt a time course. As a consequence the reactions are easier to run. Second, with this method it is easier to control the extent of the elongations in the pulse, and so the efficiency of labeling of sequences near the primer (the first 50 bases) is increased approximately 10-fold.

7 µl of 0.75 mM single-stranded M13 DNA (mGP1-2) was mixed with 1µl of complementary sequencing primer (17-mer, 0.5 pmole primer/µl) and 2 µl 5X annealing buffer (200 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 250 mM NaCl) heated at 65°C for 2 min, and slowly cooled to room temperature over 30 min. In the pulse reaction 10 µl of the above annealed mix was mixed with 1 µl dithiothreitol 0.1 M, 2 µl of 3 dNTPs (dGTP, dCTP, dTTP) 1.5 µM each, 0.5 µl [α^{35}S]dATP, (α10µM) (about 10µM, 1500 Ci/mmol, New England Nuclear) and 2 µl modified T7 DNA polymerase (0.1 mg/ml, 1000 units/ml, i.e., 0.2 µg, 2 units) and incubated at 37°C for 5 min. (The

temperature and time of incubation can be varied from 20°C-45°C and 1-60 min., respectively.)

3.5 µl aliquots of the above pulse reaction were added to each of four tubes containing the chase mixes, which were preheated to 37°C. The four tubes, labeled G, A, T, C, each contain trace amounts of either dideoxy G, A, T, C. The specific chase solutions are given below. Each tube contains 0.5 µl 5X annealing buffer (200 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 250 mM NaCl), 1 µl 4dNTPs (dGTP, dATP, dTTP, dCTP) 200 µM each, and 1.0 µl ddNTP 20 µM. Each chase reaction is incubated at 37°C for 5 min (or 20°C-45°C and 1-60 min respectively), and then 4 µl of a stop solution (95% formamide, 20 mM EDTA, 0.05% xylene-cyanol) added to each tube, and the tube placed on ice prior to running on a standard polyacrylamide sequencing gel as described above.

Example 4: Replacement of dGTP with dITP for DNA sequencing

In order to sequence through regions of compression in DNA, i.e., regions having compact secondary structure, it is common to use dITP (Mills et al., 76 Proc. Natl. Acad. Sci. 2232, 1979) or deazaguanosine triphosphate (deaza GTP, Mizusawa et al., 14 Nuc. Acid Res. 1319, 1986). We have found that both analogs function well with T7-type polymerases, especially with dITP in the presence of ssb. Preferably these reactions are performed with the above described genetically modified T7 polymerase, or the chase reaction is for 1-2 min., and/or at 20°C to reduce exonuclease degradation.

Modified T7 DNA polymerase efficiently utilizes dITP or deaza-GTP in place of dGTP. dITP is substituted for dGTP in both the pulse and chase mixes at a concentration two to five times that at which dGTP is

used. In the ddG chase mix ddGTP is still used (not ddITP).

The chase reactions using dITP are sensitive to the residual low levels (about 0.01 units) of exonuclease activity. To avoid this problem, the chase reaction times should not exceed 5 min when dITP is used. It is recommended that the four dITP reactions be run in conjunction with, rather than to the exclusion of, the four reactions using dGTP. If both dGTP and dITP are routinely used, the number of required mixes can be minimized by: (1) Leaving dGTP and dITP out of the chase mixes, which means that the four chase mixes can be used for both dGTP and dITP chase reactions. (2) Adding a high concentration of dGTP or dITP (2µl at 0.5 mM and 1-2.5 mM respectively) to the appropriate pulse mix. The two pulse mixes then each contain a low concentration of dCTP,dTTP and [α^{35}S]dATP, and a high concentration of either dGTP or dITP. This modification does not usually adversely effect the quality of the sequencing reactions, and reduces the required number of pulse and chase mixes to run reactions using both dGTP and dITP to six.

The sequencing reaction is as for example 3, except that two of the pulse mixes contain a) 3 dNTP mix for dGTP: 1.5 µM dCTP,dTTP, and 1 mM dGTP and b) 3 dNTP mix for dITP: 1.5 µM dCTP,dTTP, and 2 mM dITP. In the chase reaction dGTP is removed from the chase mixes (i.e. the chase mixes contain 30 µM dATP,dTTP and dCTP, and one of the four dideoxynucleotides at 8 µM), and the chase time using dITP does not exceed 5 min.

Deposits

Strains K38/pGP5-5/pTrx-2, K38/pTrx-2 and M13 mGP1-2 have been deposited with the ATCC and assigned numbers 67,287, 67,286, and 40,303 respectively. These deposits were made on January 13, 1987. Strain K38/pGP1-2/pGP5-6 was deposited with the ATCC. On December 4, 1987, and assigned the number 67571.

Applicants' and their assignees acknowledge their responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, 5 years after the last request for a culture, or 30 years, whichever is the longer, and its responsibility to notify the depository of the issuance of such a patent, at which time the deposits will be made irrevocably available to the public. Until that time the deposits will be made irrevocably available to the Commissioner of Patents under the terms of 37 CFR Section 1-14 and 35 USC Section 112.

Other Embodiments

Other embodiments are within the following claims.

Other uses of the modified DNA polymerases of this invention, which take advantage of their processivity, and lack of exonuclease activity, include the direct enzymatic amplification of genomic DNA sequences. This has been described, for other polymerases, by Saiki et al., 230 Science 1350, 1985; and Scharf, 233 Science 1076, 1986.

Referring to Fig. 6, enzymatic amplification of a specific DNA region entails the use of two primers which anneal to opposite strands of a double stranded DNA sequence in the region of interest, with their 3' ends directed toward one another (see dark arrows). The actual procedure involves multiple (10-40, preferably 16-20) cycles of denaturation, annealing, and DNA

synthesis. Using this procedure it is possible to amplify a specific region of human genomic DNA over 200,000 times. As a result the specific gene fragment represents about one part in five, rather than the initial one part in a million. This greatly facilitates both the cloning and the direct analysis of genomic DNA. For diagnostic uses, it can speed up the analysis from several weeks to 1-2 days.

Unlike Klenow fragment, where the amplification process is limited to fragments under two hundred bases in length, modified T7-type DNA polymerases should (preferably in conjuction with E. coli DNA binding protein, or ssb, to prevent "snapback formation of single stranded DNA) permit the amplification of DNA fragments thousands of bases in length.

The modified T7-type DNA polymerases are also suitable in standard reaction mixtures: for a) filling in 5' protruding termini of DNA fragments generated by restriction enzyme cleavage; in order to, for example, produce blunt-ended double stranded DNA from a linear DNA molecule having a single stranded region with no 3' protruding termini; b) for labeling the 3' termini of restriction fragments, for mapping mRNA start sites by S1 nuclease analysis, or sequencing DNA using the Maxam and Gilbert chemical modification procedure; and c) for in vitro mutagenesis of cloned DNA fragments. For example, a chemically synthesized primer which contains specific mismatched bases is hybridized to a DNA template, and then extended by the modified T7-type DNA polymerase. In this way the mutation becomes permanently incorporated into the synthesized strand. It is advantageous for the polymerase to synthesize from the primer through the entire length of the DNA. This

is most efficiently done using a processive DNA polymerase. Alternatively mutagenesis is performed by misincorporation during DNA synthesis (see above). This application is used to mutagenize specific regions of cloned DNA fragments. It is important that the enzyme used lack exonuclease activity. By standard reaction mixture is meant a buffered solution containing the polymerase and any necessary deoxynucleosides, or other compounds.

CLAIMS

1. A method for determining the nucleotide base sequence of a DNA molecule, comprising:

annealing said DNA molecule with a primer molecule able to hybridize to said DNA molecule;

incubating separate portions of the annealed mixture in at least four vessels, each vessel containing four different deoxynucleoside triphosphates, a processive DNA polymerase, wherein said polymerase remains bound to said DNA molecule for at least 500 bases before dissociating in an environmental condition normally used in the extension reaction of a DNA sequencing reaction, said polymerase having less than 500 units of exonuclease activity per mg of said polymerase, and one of four DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each said agent terminates DNA synthesis at a different nucleotide base, and

separating the DNA products of each incubating reaction according to their size, whereby at least a part of the nucleotide base sequence of said DNA molecule can be determined.

2. The method of claim 1 wherein said polymerase remains bound to said DNA molecule for at least 1,000 bases before dissociating.

3. The method of claim 1 or claim 2 wherein said polymerase is that polymerase in cells infected with a T7-type phage.

4. The method of claim 4 wherein said T7-type phage is T7, T3, ΦI, ΦII, H, W31, gh-1, Y, A1122 or SP6.

5. The method of claim 4 wherein said T7-type phage is T-7.

6. The method of any one of the preceding claims wherein said polymerase is unable to exhibit its processivity in a second environmental condition normally used in the pulse reaction of a DNA sequencing reaction.

7. The method of any one of the preceding claims wherein said polymerase is non-discriminating for dideoxy nucleotide analogs.

8. The method of any one of the preceding claims wherein said polymerase is a modified polymerase having less than 50 units of exonuclease activity per mg of polymerase.

9. The method of claim 8 wherein said modified polymerase has less than 1 unit of exonuclease activity per mg of polymerase.

10. The method of claim 8 wherein said modified polymerase has less than 0.1 unit of exonuclease activity per mg of polymerase.

11. The method of any one of claims 1 to 7 wherein said polymerase has no detectable exonuclease activity.

12. The method of any one of the preceding claims wherein said polymerase is able to utilize primers of 10 base pairs or more.

13. The method of any one of claims 1 to 11 wherein said polymerase is able to utilize primers of 4 base pairs or more.

14. The method of claim 13 wherein said primer comprises 4-20 base pairs and said polymerase is able to utilize primers of 4-20 base pairs.

15. The method of claim 13 or claim 14 wherein said mixture comprises T7 gene 2.5 or gene 4.

16. The method of claim 3 wherein said polymerase is non-discriminating for nucleotide analogs, and is a modified polymerase having less than 500 units of exonuclease activity per mg of polymerase

said primer is single stranded RNA or DNA containing 4-10 bases and said polymerase is able to utilize primers of 4-10 bases,

and said incubating comprises a pulse and a chase step.

17. The method of any one of claims 1 to 15 wherein said primer is single stranded DNA or RNA.

18. The method of any one of the preceding claims wherein said annealing comprises heating said DNA molecule and said primer to above 65°C, and allowing the heated mixture to cool to 0°C to 30°C.

19. The method of any one of the preceding claims wherein said incubating comprises a pulse and a chase step.

20. The method of claim 19 wherein said pulse step comprises mixing said annealed mixture with all four deoxynucleoside triphosphates and a processive DNA polymerase, wherein at least one said deoxynucleoside triphosphate is labelled and present in a limiting concentration.

21. The method of claim 20 wherein said pulse step incubation is carried out for 30 seconds to 20 minutes.

22. The method of claim 20 wherein said chase step comprises adding one said chain terminating agent to four separate aliquots of the mixture after performing said pulse step.

23. The method of claim 22 wherein said chase step incubation is carried out for 1 to 60 minutes.

24. The method of any one of the preceding claims wherein said terminating agent is a dideoxynucleotide.

25. The method of any one of the preceding claims wherein said terminating agent is a limiting level of one deoxynucleoside triphosphate.

26. The method of any one of the preceding claims wherein one said deoxynucleoside triphosphate is chosen from dITP or deazaguanosine.

27. The method of any one of claims 1 to 18 wherein said primer is labelled prior to said annealing step.

28. The method of claim 27 wherein the incubating comprises a chase step.

29. The method of claim 27 wherein said primer is fluorescently labelled.

30. A kit for DNA sequencing comprising:

a processive DNA polymerase, wherein said polymerase remains bound to a DNA molecule for at least 500 bases before dissociating, said polymerase having less than 500 units of exonuclease activity per mg of polymerase, said polymerase being able to exhibit its processivity in an environmental condition normally used in the extension reaction of a DNA sequencing reaction, and

a reagent necessary for said sequencing, selected from

(a) dITP and

(b) a chain terminating agent.

31. The kit of claim 30 wherein said reagent is dITP.

32. The kit of claim 30 or claim 31 wherein said polymerase is unable to exhibit its processivity in a second environmental condition normally used in the pulse reaction of DNA sequencing reaction.

33. A method of producing active T7-type DNA polymerase from cloned fragments, comprising placing the genes encoding said polymerase under the control of non-leaky promoters in a single cell, inducing expression of said genes when said cell is in logarithmic growth phase, or stationary phase, and isolating said polymerase from said cell.

34. The method of claim 33 wherein one of said genes is under the control of a promoter requiring T7 RNA polymerase for expression.

35. The method of claim 33 wherein said polymerase is active T7 DNA polymerase produced from two cloned genes, one said gene being under the control of a promoter requiring T7 RNA polymerase for expression.

36. A method of purifying T7 DNA polymerase from cells comprising a vector from which said polymerase is expressed, comprising the steps of: lysing said cells, and passing said polymerase over a sizing column, a DE52 DEAE column, a phosphocellulose column, and a hydroxy apatite column.

37. The method of claim 36 wherein prior to said passing step said method comprises precipitating said polymerase with ammonium sulfate.

38. The method of claim 36 further comprising the steps of passing said polymerase over a Sephadex DEAE A-50 column.

39. The method of claim 36 wherein said sizing column is a Sephadex G150 column.

40. A method of inactivating exonuclease activity in a DNA polymerase solution produced by recombinant DNA techniques, comprising: incubating said solution in a vessel containing oxygen, a reducing agent and a transition metal.

41. A gene encoding a T7-type DNA polymerase, said gene being genetically modified to reduce the activity of naturally occurring exonuclease activity.

42. A T7-type DNA polymerase produced from the gene of claim 41.

43. The gene of claim 41 having a modified His residue.

44. The gene of claim 43, said His residue being His-123 of T7 DNA polymerase.

45. The gene of claim 43 or claim 44 wherein said His residue is deleted.

46. A T-7 type DNA polymerase produced from the gene of claim 44 or 45.

47. The gene of claim 41 wherein amino acids 118 to 123 are deleted from the gene encoding T7-type DNA polymerase.

48. A method for producing blunt-ended double stranded DNA from a linear DNA molecule having a single stranded region, wherein the 3' end of said molecule is double stranded and has no 3' protruding termini, comprising

incubating said DNA molecule with a processive DNA polymerase essentially free from naturally occurring exonuclease activity.

49. A method of amplification of a DNA sequence comprising annealing a first and second primer to opposite strands of a double stranded DNA sequence and incubating the annealed mixture with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase, wherein said first and second primers anneal to opposite strands of said DNA sequence.

50. The method of claim 49 wherein said first and second primers have their 3' ends directed toward each other after annealing.

51. The method of claim 49 or claim 50 wherein said method further comprises, after said incubation step, denaturing the resulting DNA, annealing said first and second primers to said resulting DNA and incubating the annealed mixture with said polymerase.

52. The method of claim 51 wherein said cycle of denaturing, annealing and incubating is repeated from 10 to 40 times.

53. The method of any one of claims 49 to 52 wherein said exonuclease activity is less than 1 unit per mg of polymerase.

54. A method for labelling the 3' end of a DNA fragment comprising incubating said DNA fragment with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase and a labelled deoxynucleotide.

55. A method for _in vitro_ mutagenesis of a cloned DNA fragment comprising providing a primer and a template, said primer and said template having a specific mismatched base, and extending said primer with a processive modified DNA polymerase.

56. A method for _in vitro_ mutagenesis of a cloned DNA fragment comprising providing said cloned fragment and synthesizing a DNA strand using a processive DNA polymerase, having less than 1 unit of exonuclease activity per mg of polymerase, under conditions which cause misincorporation of a nucleotide base.

57. A kit comprising a plurality of at least 25 oligomers suitable for DNA sequencing, each said oligomer being contained in a separate container and having a different base sequence of less than 15 bases in length.

58. The kit of claim 57 wherein said oligomers each comprise between 6-9 bases inclusive.

FIGURE 1

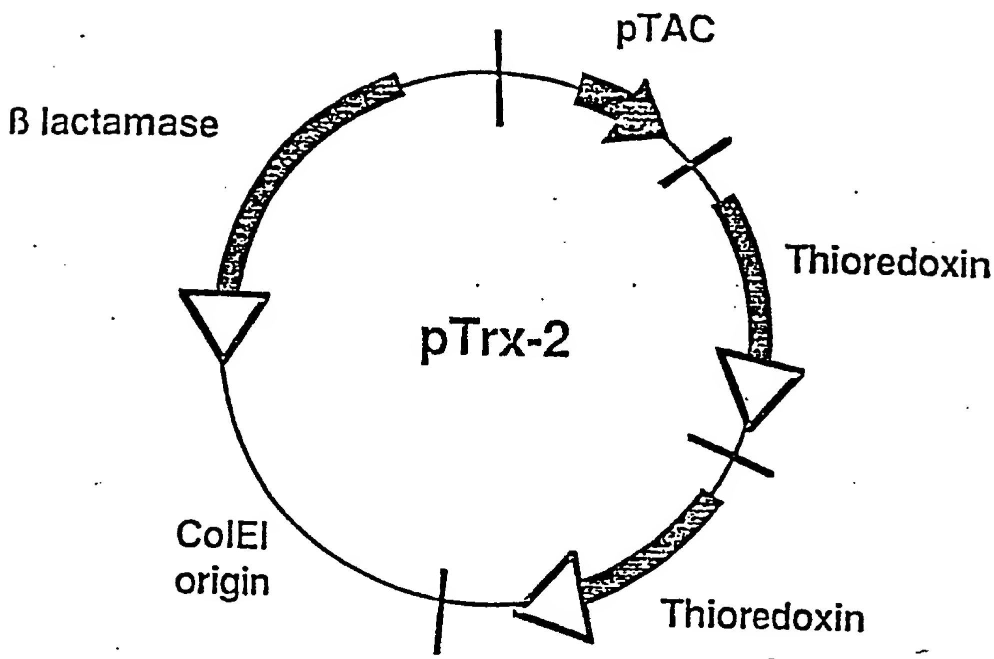

pTAC
ß lactamase
Thioredoxin
pTrx-2
ColEI
origin
Thioredoxin

FIGURE 2

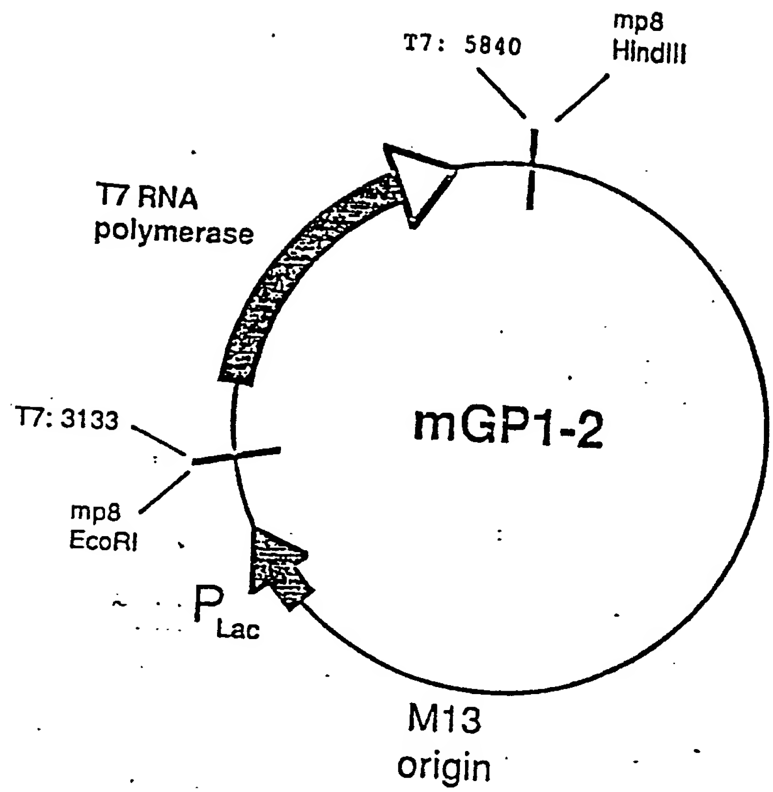

T7: 5840
mp8
HindIII
T7 RNA
polymerase
T7: 3133
mp8
EcoRI
P_{Lac}
mGP1-2
M13
origin

FIGURE 3

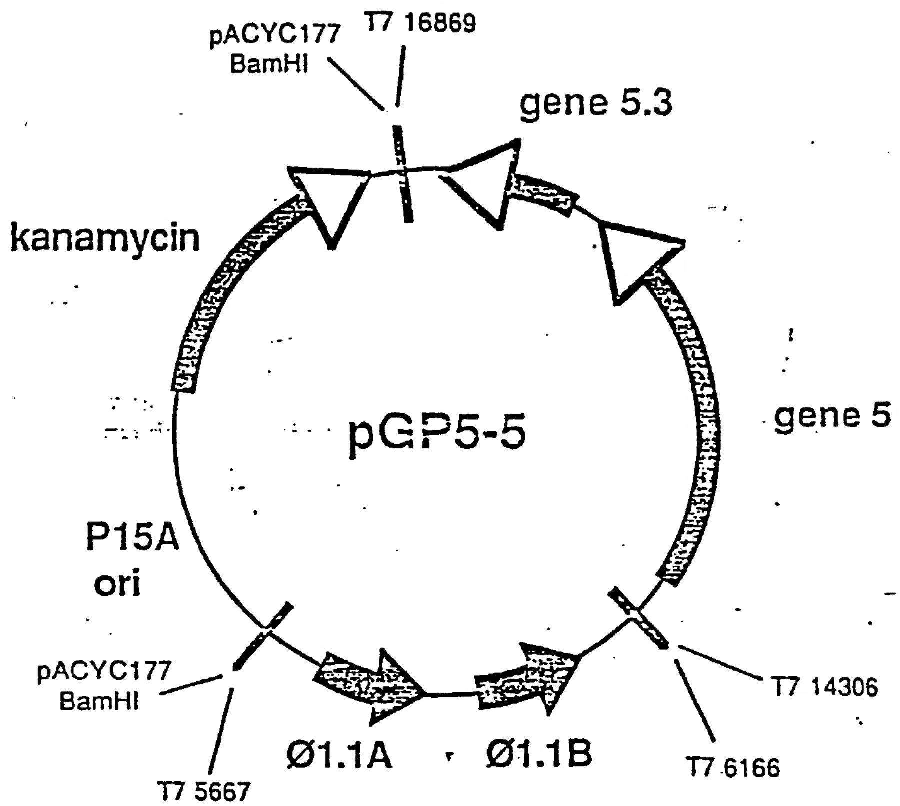
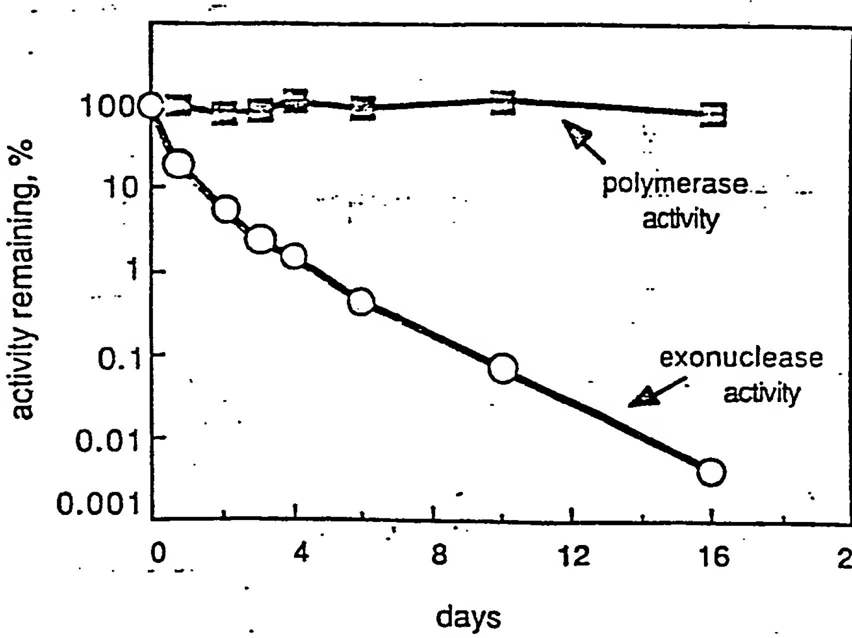

pACYC177
BamHI
T7 16869
gene 5.3
kanamycin
gene 5
pGP5-5
P15A
ori
pACYC177
BamHI
T7 14306
T7 5667
Ø1.1A
Ø1.1B
T7 6166

FIGURE 4



100
10
1
0.1
0.01
0.001
activity remaining, %
0
4
8
12
16
20
days
polymerase activity
exonuclease activity

FIGURE 5

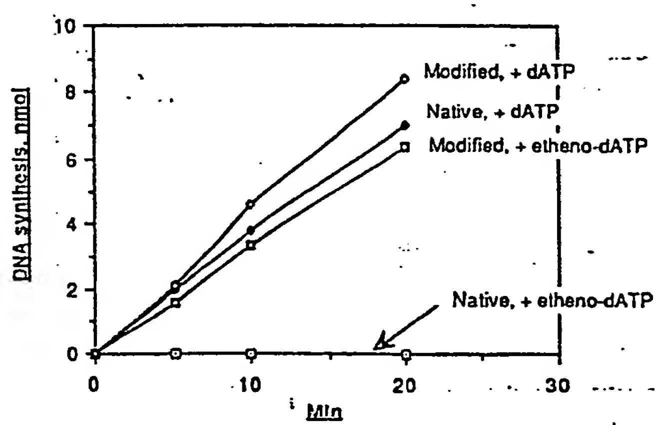

Modified, + dATP
Native, + dATP
Modified, + etheno-dATP
Native, + etheno-dATP
DNA synthesis, nmol
Min
0
2
4
6
8
10
0
10
20
30

FIGURE 6

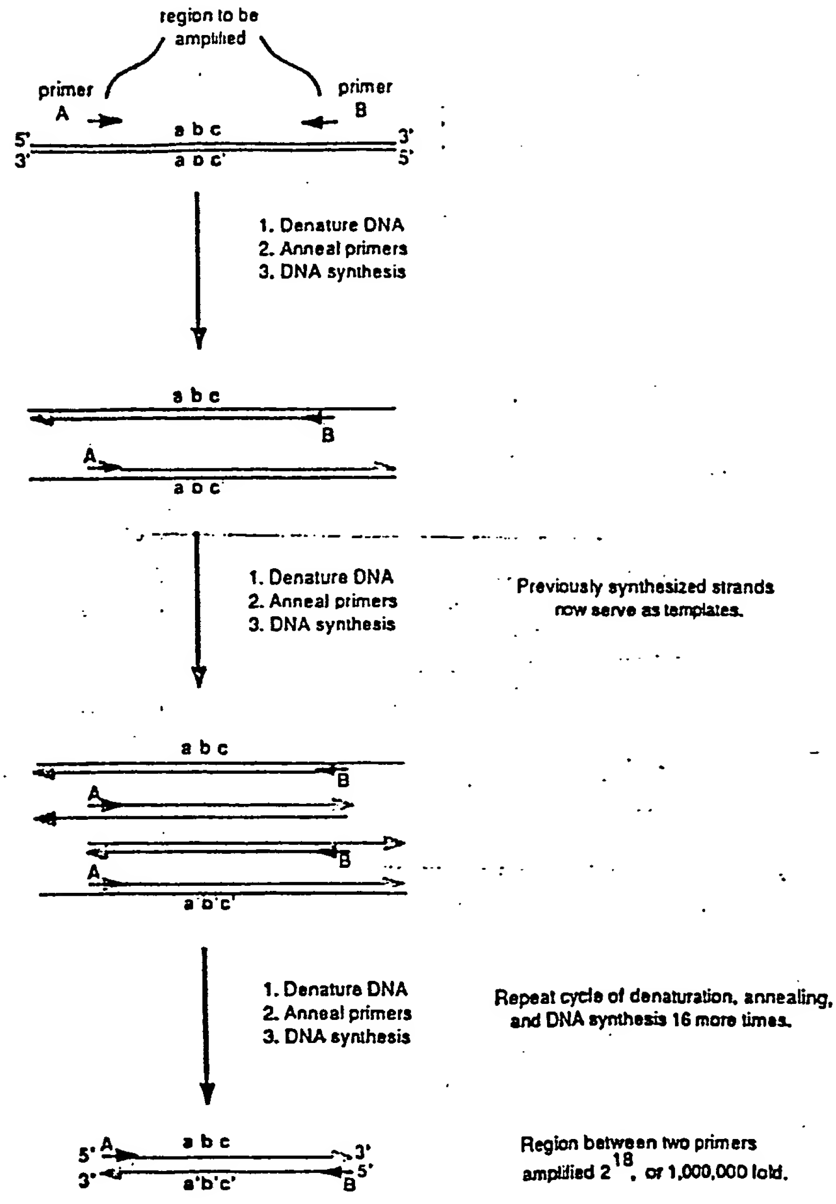

region to be amplified
primer A
primer B
5'
3'
a b c
a' b' c'
3'
5'
1. Denature DNA
2. Anneal primers
3. DNA synthesis
a b c
B
A
a' b' c'
1. Denature DNA
2. Anneal primers
3. DNA synthesis
Previously synthesized strands now serve as templates.
a b c
B
A
B
A
a' b' c'
1. Denature DNA
2. Anneal primers
3. DNA synthesis
Repeat cycle of denaturation, annealing, and DNA synthesis 16 more times.
5' A
a b c
3'
3'
a'b'c'
B 5'
Region between two primers amplified 2^18, or 1,000,000 fold.

FIGURE 7

10	20	30	40	50
TTCTTCTCAT	GTTTGACAGC	TTATCATCGA	CTGCACGGTG	CACCAATGCT
60	70	80	90	100
TCTGGCGTCA	GGCAGCCATC	GGAAGCTGTG	GTATGGCTGT	GCAGGTCGTA
110	120	130	140	150
AATCACTGCA	TAATTCGTGT	CGCTCAAGGC	GCACTCCCGT	TCTGGATAAT
160	170	180	190	200
GTTTTTTGCG	CCGACATCAT	AACGGTTCTG	GCAAATATTC	TGAAATGAGC
210	220	230	240	250
TGTTGACAAT	TAATCATCGG	CTCGTATAAT	GTGTGGAATT	GTGAGCGGAT
260	270	280	290	300
AACAATTTCA	CACAGGAAAC	AGGGGATCCG	TCAACCTTTA	GTTGGTTAAT
310	320	330	340	350
GTTACACCAA	CAACGAAACC	AACACGCCAG	GCTTATTCCT	GTGGAGTTAT
360	370	380	390	400
ATATGAGCGA	TAAAATTATT	CACCTGACTG	ACGACAGTTT	TGACACGGAT
410	420	430	440	450
GTACTCAAAG	CGGACGGGGC	GATCCTCGTC	GATTTCTGGG	CAGAGTGGTG
460	470	480	490	500
CGGTCCGTGC	AAGATGATCG	CCCCGATTCT	GGATGAAATC	GCTGACGAAT

FIGURE 7 (continued)

510	520	530	540	550
ATCAGGGCAA	ACTGACCGTT	GCAAAACTGA	ACATCGATCA	AAACCCTGGT
560	570	580	590	600
ACTGCGCCGA	AATATGGCAT	CCGTGGTATC	CCGACTCTGC	TGCTGTTCAA
610	620	630	640	650
AAACGGTGAA	GTGGCGGCAA	CCAAAGTGGG	TGCACTGTCT	AAAGGTCAGT
660	670	680	690	700
TGAAAGAGTT	CCTCGACGCT	AACCTGGCGT	AAGGGAATTT	CATGTTCGGG
710	720	730	740	750
TGCCCCGTCG	CTAAAAACTG	GACGCCCGGC	GTGAGTCATG	CTAACTTAGT
760	770	780	790	800
GTTGACGGAT	CCCCGGGGAT	CCGTCAACCT	TTAGTTGGTT	AATGTTACAC
810	820	830	840	850
CAACAACGAA	ACCAACACGC	CAGGCTTATT	CCTGTGGAGT	TATATATGAG
860	870	880	890	900
CGATAAAATT	ATTCACCTGA	CTGACGACAG	TTTTGACACG	GATGTACTCA
910	920	930	940	950
AAGCGGACGG	GGCGATCCTC	GTCGATTTCT	GGGCAGAGTG	GTGCGGTCCG
960	970	980	990	1000
TGCAAGATGA	TCGCCCCGAT	TCTGGATGAA	ATCGCTGACG	AATATCAGGG
1010	1020	1030	1040	1050
CAAACTGACC	GTTGCAAAAC	TGAACATCGA	TCAAAACCCT	GGTACTGCGC
1060	1070	1080	1090	1100
CGAAATATGG	CATCCGTGGT	ATCCCGACTC	TGCTGCTGTT	CAAAAACGGT
1110	1120	1130	1140	1150
GAAGTGGCGG	CAACCAAAGT	GGGTGCACTG	TCTAAAGGTC	AGTTGAAAGA
1160	1170	1180	1190	1200
GTTCCTCGAC	GCTAACCTGG	CGTAAGGGAA	TTTCATGTTC	GGGTGCCCCG
1210	1220	1230	1240	1250
TCGCTAAAAA	CTGGACGCCC	GGCGTGAGTC	ATGCTAACTT	AGTGTTGACG
1260	1270	1280	1290	1300
GATCCCCCTG	CCTCGCGCGT	TTCGGTGATG	ACGGTGAAAA	CCTCTGACAC
1310	1320	1330	1340	1350
ATGCAGCTCC	CGGAGACGGT	CACAGCTTGT	CTGTAAGCGG	ATGCCGGGAG
1360	1370	1380	1390	1400
CAGACAAGCC	CGTCAGGGCG	CGTCAGCGGG	TGTTGGCGGG	TGTCGGGGCG
1410	1420	1430	1440	1450
CAGCCATGAC	CCAGTCACGT	AGCGATAGCG	GAGTGTATAC	TGGCTTAACT
1460	1470	1480	1490	1500
ATGCGGCATC	AGAGCAGATT	GTACTGAGAG	TGCACCATAT	GCGGTGTGAA
1510	1520	1530	1540	1550
ATACCGCACA	GATGCGTAAG	GAGAAAATAC	CGCATCAGGC	GCTCTTCCGC
1560	1570	1580	1590	1600
TTCCTCGCTC	ACTGACTCGC	TGCGCTCGGT	CGTTCGGCTG	CGGCGAGCGG
1610	1620	1630	1640	1650
TATCAGCTCA	CTCAAAGGCG	GTAATACGGT	TATCCACAGA	ATCAGGGGAT
1660	1670	1680	1690	1700
AACGCAGGAA	AGAACATGTG	AGCAAAAGGC	CAGCAAAAGG	CCAGGAACCG
1710	1720	1730	1740	1750
TAAAAAGGCC	GCGTTGCTGG	CGTTTTTCCA	TAGGCTCCGC	CCCCCTGACG
1760	1770	1780	1790	1800
AGCATCACAA	AAATCGACGC	TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA
1810	1820	1830	1840	1850
CTATAAAGAT	ACCAGGCGTT	TCCCCCTGGA	AGCTCCCTCG	TGCGCTCTCC

FIGURE 7 (continued)

1860	1870	1880	1890	1900
TGTTCCGACC	CTGCCGCTTA	CCGGATACCT	GTCCGCCTTT	CTCCCTTCGG
1910	1920	1930	1940	1950
GAAGCGTGGC	GCTTTCTCAA	TGCTCACGCT	GTAGGTATCT	CAGTTCGGTG
1960	1970	1980	1990	2000
TAGGTCGTTC	GCTCCAAGCT	GGGCTGTGTG	CACGAACCCC	CCGTTCAGCC
2010	2020	2030	2040	2050
CGACCGCTGC	GCCTTATCCG	GTAACTATCG	TCTTGAGTCC	AACCCGGTAA
2060	2070	2080	2090	2100
GACACGACTT	ATCGCCACTG	GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA
2110	2120	2130	2140	2150
GCGAGGTATG	TAGGCGGTGC	TACAGAGTTC	TTGAAGTGGT	GGCCTAACTA
2160	2170	2180	2190	2200
CGGCTACACT	AGAAGGACAG	TATTTGGTAT	CTGCGCTCTG	CTGAAGCCAG
2210	2220	2230	2240	2250
TTACCTTCGG	AAAAAGAGTT	GGTAGCTCTT	GATCCGGCAA	ACAAACCACC
2260	2270	2280	2290	2300
GCTGGTAGCG	GTGGTTTTTT	TGTTTGCAAG	CAGCAGATTA	CGCGCAGAAA
2310	2320	2330	2340	2350
AAAAGGATCT	CAAGAAGATC	CTTTGATCTT	TTCTACGGGG	TCTGACGCTC
2360	2370	2380	2390	2400
AGTGGAACGA	AAACTCACGT	TAAGGGATTT	TGGTCATGAG	ATTATCAAAA
2410	2420	2430	2440	2450
AGGATCTTCA	CCTAGATCCT	TTTAAATTAA	AAATGAAGTT	TTAAATCAAT
2460	2470	2480	2490	2500
CTAAAGTATA	TATGAGTAAA	CTTGGTCTGA	CAGTTACCAA	TGCTTAATCA
2510	2520	2530	2540	2550
GTGAGGCACC	TATCTCAGCG	ATCTGTCTAT	TTCGTTCATC	CATAGTTGCC
2560	2570	2580	2590	2600
TGACTCCCCG	TCGTGTAGAT	AACTACGATA	CGGGAGGGCT	TACCATCTGG
2610	2620	2630	2640	2650
CCCCAGTGCT	GCAATGATAC	CGCGAGACCC	ACGCTCACCG	GCTCCAGATT
2660	2670	2680	2690	2700
TATCAGCAAT	AAACCAGCCA	GCCGGAAGGG	CCGAGCGCAG	AAGTGGTCCT
2710	2720	2730	2740	2750
GCAACTTTAT	CCGCCTCCAT	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG
2760	2770	2780	2790	2800
AGTAAGTAGT	TCGCCAGTTA	ATAGTTTGCG	CAACGTTGTT	GCCATTGCTG
2810	2820	2830	2840	2850
CAGGCATCGT	GGTGTCACGC	TCGTCGTTTG	GTATGGCTTC	ATTCAGCTCC
2860	2870	2880	2890	2900
GGTTCCCAAC	GATCAAGGCG	AGTTACATGA	TCCCCCATGT	TGTGCAAAAA
2910	2920	2930	2940	2950
AGCGGTTAGC	TCCTTCGGTC	CTCCGATCGT	TGTCAGAAGT	AAGTTGGCCG
2960	2970	2980	2990	3000
CAGTGTTATC	ACTCATGGTT	ATGGCAGCAC	TGCATAATTC	TCTTACTGTC
3010	3020	3030	3040	3050
ATGCCATCCG	TAAGATGCTT	TTCTGTGACT	GGTGAGTACT	CAACCAAGTC
3060	3070	3080	3090	3100
ATTCTGAGAA	TAGTGTATGC	GGCGACCGAG	TTGCTCTTGC	CCGGCGTCAA
3110	3120	3130	3140	3150
CACGGGATAA	TACCGCGCCA	CATAGCAGAA	CTTTAAAAGT	GCTCATCATT
3160	3170	3180	3190	3200
GGAAAACGTT	CTTCGGGGCG	AAAACTCTCA	AGGATCTTAC	CGCTGTTGAG

FIGURE 7 (continued)

3210	3220	3230	3240	3250
ATCCAGTTCG	ATGTAACCCA	CTCGTGCACC	CAACTGATCT	TCAGCATCTT
3260	3270	3280	3290	3300
TTACTTTCAC	CAGCGTTTCT	GGGTGAGCAA	AAACAGGAAG	GCAAAATGCC
3310	3320	3330	3340	3350
GCAAAAAAGG	GAATAAGGGC	GACACGGAAA	TGTTGAATAC	TCATACTCTT
3360	3370	3380	3390	3400
CCTTTTTCAA	TATTATTGAA	GCATTTATCA	GGGTTATTGT	CTCATGAGCG
3410	3420	3430	3440	3450
GATACATATT	TGAATGTATT	TAGAAAAATA	AACAAATAGG	GGTTCCGCGC
3460	3470	3480	3490	3500
ACATTTCCCC	GAAAAGTGCC	ACCTGACGTC	TAAGAAACCA	TTATTATCAT
3510	3520	3530	3540	3550
GACATTAACC	TATAAAAATA	GGCGTATCAC	GAGGCCCTTT	CGTCTTCAAG

AA

FIGURE 8

10	20	30	40	50
GTTGACACAT	ATGAGTCTTG	TGATGTACTG	GCTGATTTCT	ACGACCAGTT
60	70	80	90	100
CGCTGACCAG	TTGCACGAGT	CTCAATTGGA	CAAAATGCCA	GCACTTCCGG
110	120	130	140	150
CTAAAGGTAA	CTTGAACCTC	CGTGACATCT	TAGAGTCGGA	CTTCGCGTTC
160	170	180	190	200
GCGTAACGCC	AAATCAATAC	GACTCACTAT	AGAGGGACAA	ACTCAAGGTC
210	220	230	240	250
ATTCGCAAGA	GTGGCCTTTA	TGATTGACCT	TCTTCCGGTT	AATACGACTC
260	270	280	290	300
ACTATAGGAG	AACCTTAAGG	TTTAACTTTA	AGACCCTTAA	GTGTTAATTA
310	320	330	340	350
GAGATTTAAA	TTAAAGAATT	ACTAAGAGAG	GACTTTAAGT	ATGCGTAACT
360	370	380	390	400
TCGAAAAGAT	GACCAAACGT	TCTAACCGTA	ATGCTCGTGA	CTTCGAGGCA
410	420	430	440	450
ACCAAAGGTC	GCAAGTTGAA	TAAGACTAAG	CGTGACCGCT	CTCACAAGCG
460	470	480	490	500
TAGCTGGGAG	GGTCAGTAAG	ATGGGACGTT	TATATAGTGG	TAATCTGGCA
510	520	530	540	550
CCGGATCCGG	TATGAAGAGA	TTGTTAAGTC	ACGATAATCA	ATAGGAGAAA
560	570	580	590	600
TCAATATGAT	CGTTTCTGAC	ATCGAAGCTA	ACGCCCTCTT	AGAGAGCGTC

FIGURE 8 (continued)

610	620	630	640	650
ACTAAGTTCC	ACTGCGGGGT	TATCTACGAC	TACTCCACCG	CTGAGTACGT
660	670	680	690	700
AAGCTACCGT	CCGAGTGACT	TCGGTGCGTA	TCTGGATGCG	CTGGAAGCCG
710	720	730	740	750
AGGTTGCACG	AGGCGGTCTT	ATTGTGTTCC	ACAACGGTCA	CAAGTATGAC
760	770	780	790	800
GTTCCTGCAT	TGACCAAACT	GGCAAAGTTG	CAATTGAACC	GAGAGTTCCA
810	820	830	840	850
CCTTCCTCGT	GAGAACTGTA	TTGACACCCT	TGTGTTGTCA	CGTTTGATTC
860	870	880	890	900
ATTCCAACCT	CAAGGACACC	GATATGGGTC	TTCTGCGTTC	CGGCAAGTTG
910	920	930	940	950
CCCGGAAAAC	GCTTTGGGTC	TCACGCTTTG	GAGGCGTGGG	GTTATCGCTT
960	970	980	990	1000
AGGCGAGATG	AAGGGTGAAT	ACAAAGACGA	CTTTAAGCGT	ATGCTTGAAG
1010	1020	1030	1040	1050
AGCAGGGTGA	AGAATACGTT	GACGGAATGG	AGTGGTGGAA	CTTCAACGAA
1060	1070	1080	1090	1100
GAGATGATGG	ACTATAACGT	TCAGGACGTT	GTGGTAACTA	AAGCTCTCCT
1110	1120	1130	1140	1150
TGAGAAGCTA	CTCTCTGACA	AACATTACTT	CCCTCCTGAG	ATTGACTTTA
1160	1170	1180	1190	1200
CGGACGTAGG	ATACACTACG	TTCTGGTCAG	AATCCCTTGA	GGCCGTTGAC
1210	1220	1230	1240	1250
ATTGAACATC	GTGCTGCATG	GCTGCTCGCT	AAACAAGAGC	GCAACGGGTT
1260	1270	1280	1290	1300
CCCGTTTGAC	ACAAAAGCAA	TCGAAGAGTT	GTACGTAGAG	TTAGCTGCTC
1310	1320	1330	1340	1350
GCCGCTCTGA	GTTGCTCCGT	AAATTGACCG	AAACGTTCGG	CTCGTGGTAT
1360	1370	1380	1390	1400
CAGCCTAAAG	GTGGCACTGA	GATGTTCTGC	CATCCGCGAA	CAGGTAAGCC
1410	1420	1430	1440	1450
ACTACCTAAA	TACCCTCGCA	TTAAGACACC	TAAAGTTGGT	GGTATCTTTA
1460	1470	1480	1490	1500
AGAAGCCTAA	GAACAAGGCA	CAGCGAGAAG	GCCGTGAGCC	TTGCGAACTT
1510	1520	1530	1540	1550
GATACCCGCG	AGTACGTTGC	TGGTGCTCCT	TACACCCCAG	TTGAACATGT
1560	1570	1580	1590	1600
TGTGTTTAAC	CCTTCGTCTC	GTGACCACAT	TCAGAAGAAA	CTCCAAGAGG
1610	1620	1630	1640	1650
CTGGGTGGGT	CCCGACCAAG	TACACCGATA	AGGGTGCTCC	TGTGGTGGAC
1660	1670	1680	1690	1700
GATGAGGTAC	TCGAAGGAGT	ACGTGTAGAT	GACCCTGAGA	AGCAAGCCGC
1710	1720	1730	1740	1750
TATCGACCTC	ATTAAAGAGT	ACTTGATGAT	TCAGAAGCGA	ATCGGACAGT
1760	1770	1780	1790	1800
CTGCTGAGGG	AGACAAAGCA	TGGCTTCGTT	ATGTTGCTGA	GGATGGTAAG
1810	1820	1830	1840	1850
ATTCATGGTT	CTGTTAACCC	TAATGGAGCA	GTTACGGGTC	GTGCGACCCA
1860	1870	1880	1890	1900
TGCGTTCCCA	AACCTTGCGC	AAATTCCGGG	TGTACGTTCT	CCTTATGGAG
1910	1920	1930	1940	1950
AGCAGTGTCG	CGCTGCTTTT	GGCGCTGAGC	ACCATTTGGA	TGGGATAACT

FIGURE 8 (continued)

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      1960       1970       1980       1990       2000
GGTAAGCCTT GGGTTCAGGC TGGCATCGAC GCATCCGGTC TTGAGCTACG
      2010       2020       2030       2040       2050
CTGCTTGGCT CACTTCATGG CTCGCTTTGA TAACGGCGAG TACGCTCACG
      2060       2070       2080       2090       2100
AGATTCTTAA CGGCGACATC CACACTAAGA ACCAGATAGC TGCTGAACTA
      2110       2120       2130       2140       2150
CCTACCCGAG ATAACGCTAA GACGTTCATC TATGGGTTCC TCTATGGTGC
      2160       2170       2180       2190       2200
TGGTGATGAG AAGATTGGAC AGATTGTTGG TGCTGGTAAA GAGCGCGGTA
      2210       2220       2230       2240       2250
AGGAACTCAA GAAGAAATTC CTTGAGAACA CCCCCGCGAT TGCAGCACTC
      2260       2270       2280       2290       2300
CGCGAGTCTA TCCAACAGAC ACTTGTCGAG TCCTCTCAAT GGGTAGCTGG
      2310       2320       2330       2340       2350
TGAGCAACAA GTCAAGTGGA AACGCCGCTG GATTAAAGGT CTGGATGGTC
      2360       2370       2380       2390       2400
GTAAGGTACA CGTTCGTAGT CCTCACGCTG CCTTGAATAC CCTACTGCAA
      2410       2420       2430       2440       2450
TCTGCTGGTG CTCTCATCTG CAAACTGTGG ATTATCAAGA CCGAAGAGAT
      2460       2470       2480       2490       2500
GCTCGTAGAG AAAGGCTTGA AGCATGGCTG GGATGGGGAC TTTGCGTACA
      2510       2520       2530       2540       2550
TGGCATGGGT ACATGATGAA ATCCAAGTAG GCTGCCGTAC CGAAGAGATT
      2560       2570       2580       2590       2600
GCTCAGGTGG TCATTGAGAC CGCACAAGAA GCGATGCGCT GGGTTGGAGA
      2610       2620       2630       2640       2650
CCACTGGAAC TTCCGGTGTC TTCTGGATAC CGAAGGTAAG ATGGGTCCTA
      2660       2670       2680       2690       2700
ATTGGGCGAT TTGCCACTGA TACAGGAGGC TACTCATGAA CGAAAGACAC
      2710       2720       2730       2740       2750
TTAACAGGTG CTGCTTCTGA AATGCTAGTA GCCTACAAAT TTACCAAAGC
      2760       2770       2780       2790       2800
TGGGTACACT GTCTATTACC CTATGCTGAC TCAGAGTAAA GAGGACTTGG
      2810       2820       2830       2840       2850
TTGTATGTAA GGATGGTAAA TTTAGTAAGG TTCAGGTTAA AACAGCCACA
      2860       2870       2880       2890       2900
ACGGTTCAAA CCAACACAGG AGATGCCAAG CAGGTTAGGC TAGGTGGATG
      2910       2920       2930       2940       2950
CGGTAGGTCC GAATATAAGG ATGGAGACTT TGACATTCTT GCGGTTGTGG
      2960       2970       2980       2990       3000
TTGACGAAGA TGTGCTTATT TTCACATGGG ACGAAGTAAA AGGTAAGACA
      3010       3020       3030       3040       3050
TCCATGTGTG TCGGCAAGAG AAACAAAGGC ATAAAACTAT AGGAGAAATT
      3060       3070       3080
ATTATGGCTA TGACAAAGAA ATTTCCGGAT C
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FIGURE 9

10	20	30	40	50
AATGCTACTA	CTATTAGTAG	AATTGATGCC	ACCTTTTCAG	CTCGCGCCCC
60	70	80	90	100
AAATGAAAAT	ATAGCTAAAC	AGGTTATTGA	CCATTTGCGA	AATGTATCTA
110	120	130	140	150
ATGGTCAAAC	TAAATCTACT	CGTTCGCAGA	ATTGGGAATC	AACTGTTACA
160	170	180	190	200
TGGAATGAAA	CTTCCAGACA	CCGTACTTTA	GTTGCATATT	TAAAACATGT
210	220	230	240	250
TGAGCTACAG	CACCAGATTC	AGCAATTAAG	CTCTAAGCCA	TCCGCAAAAA
260	270	280	290	300
TGACCTCTTA	TCAAAAGGAG	CAATTAAAGG	TACTCTCTAA	TCCTGACCTG
310	320	330	340	350
TTGGAGTTTG	CTTCCGGTCT	GGTTCGCTTT	GAAGCTCGAA	TTAAAACGCG
360	370	380	390	400
ATATTTGAAG	TCTTTCGGGC	TTCCTCTTAA	TCTTTTTGAT	GCAATCCGCT
410	420	430	440	450
TTGCTTCTGA	CTATAATAGT	CAGGGTAAAG	ACCTGATTTT	TGATTTATGG
460	470	480	490	500
TCATTCTCGT	TTTCTGAACT	GTTTAAAGCA	TTTGAGGGGG	ATTCAATGAA
510	520	530	540	550
TATTTATGAC	GATTCCGCAG	TATTGGACGC	TATCCAGTCT	AAACATTTTA
560	570	580	590	600
CTATTACCCC	CTCTGGCAAA	ACTTCTTTTG	CAAAAGCCTC	TCGCTATTTT
610	620	630	640	650
GGTTTTTATC	GTCGTCTGGT	AAACGAGGGT	TATGATAGTG	TTGCTCTTAC
660	670	680	690	700
TATGCCTCGT	AATTCCTTTT	GGCGTTATGT	ATCTGCATTA	GTTGAATGTG
710	720	730	740	750
GTATTCCTAA	ATCTCAACTG	ATGAATCTTT	CTACCTGTAA	TAATGTTGTT
760	770	780	790	800
CCGTTAGTTC	GTTTTATTAA	CGTAGATTTT	TCTTCCCAAC	GTCCTGACTG
810	820	830	840	850
GTATAATGAG	CCAGTTCTTA	AAATCGCATA	AGGTAATTCA	CAATGATTAA
860	870	880	890	900

FIGURE 9 (continued)

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AGTTGAAATT AAACCATCTC AAGCCCAATT TACTACTCGT TCTGGTGGTT
       910        920        930        940        950
CTCGTCAGGG CAAGCCTTAT TCACTGAATG AGCAGCTTTG TTACGTTGAT
       960        970        980        990       1000
TTGGGTAATG AATATCCGGT TCTTGTCAAG ATTACTCTTG ATGAAGGTCA
      1010       1020       1030       1040       1050
GCCAGCCTAT GCGCCTGGTC TGTACACCGT TCATCTGTCC TCTTTCAAAG
      1060       1070       1080       1090       1100
TTGGTCAGTT CGGTTCCCTT ATGATTGACC GTCTGCGCCT CGTTCCGGCT
      1110       1120       1130       1140       1150
AAGTAACATG GAGCAGGTCG CGGATTTCGA CACAATTTAT CAGGCGATGA
      1160       1170       1180       1190       1200
TACAAATCTC CGTTGTACTT TGTTTCGCGC TTGGTATAAT CGCTGGGGGT
      1210       1220       1230       1240       1250
CAAAGATGAG TGTTTTAGTG TATTCTTTCG CCTCTTTCGT TTTAGGTTGG
      1260       1270       1280       1290       1300
TGCCTTCGTA GTGGCATTAC GTATTTTACC CGTTTAATGC AAACTTCCTC
      1310       1320       1330       1340       1350
ATGAAAAAGT CTTTAGTCCT CAAAGCCTCT GTAGCCGTTG CTACCCTCGT
      1360       1370       1380       1390       1400
TCCGATGCTG TCTTTCGCTG CTGAGGGTGA CGATCCCGCA AAAGCGGCCT
      1410       1420       1430       1440       1450
TTAACTCCCT GCAAGCCTCA GCGACCGAAT ATATCGGTTA TGCGTGGGCG
      1460       1470       1480       1490       1500
ATGGTTGTTG TCATTGTCGG CGCAACTATC GGTATCAAGC TGTTTAAGAA
      1510       1520       1530       1540       1550
ATTCACCTCG AAAGCAAGCT GATAAACCGA TACAATTAAA GGCTCCTTTT
      1560       1570       1580       1590       1600
GGAGCCTTTT TTTTTGGAGA TTTTCAACGT GAAAAAATTA TTATTCGCAA
      1610       1620       1630       1640       1650
TTCCTTTAGT TGTTCCTTTC TATTCTCACT CCGCTGAAAC TGTTGAAAGT
      1660       1670       1680       1690       1700
TGTTTAGCAA AACCCCATAC AGAAAATTCA TTTACTAACG TCTGGAAAGA
      1710       1720       1730       1740       1750
CGACAAAACT TTAGATCGTT ACGCTAACTA TGAGGGTTGT CTGTGGAATG
      1760       1770       1780       1790       1800
CTACAGGCGT TGTAGTTTGT ACTGGTGACG AAACTCAGTG TTACGGTACA
      1810       1820       1830       1840       1850
TGGGTTCCTA TTGGGCTTGC TATCCCTGAA AATGAGGGTG GTGGCTCTGA
      1860       1870       1880       1890       1900
GGGTGGCGGT TCTGAGGGTG GCGGTTCTGA GGGTGGCGGT ACTAAACCTC
      1910       1920       1930       1940       1950
CTGAGTACGG TGATACACCT ATTCCGGGCT ATACTTATAT CAACCCTCTC
      1960       1970       1980       1990       2000
GACGGCACTT ATCCGCCTGG TACTGAGCAA AACCCCGCTA ATCCTAATCC
      2010       2020       2030       2040       2050
TTCTCTTGAG GAGTCTCAGC CTCTTAATAC TTTCATGTTT CAGAATAATA
      2060       2070       2080       2090       2100
GGTTCCGAAA TAGGCAGGGG GCATTAACTG TTTATACGGG CACTGTTACT
      2110       2120       2130       2140       2150
CAAGGCACTG ACCCCGTTAA AACTTATTAC CAGTACACTC CTGTATCATC
      2160       2170       2180       2190       2200
AAAAGCCATG TATGACGCTT ACTGGAACGG TAAATTCAGA GACTGCGCTT
      2210       2220       2230       2240       2250
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FIGURE 9 (continued)

TCCATTCTGG	CTTTAATGAA	GATCCATTCG	TTTGTGAATA	TCAAGGCCAA
2260	2270	2280	2290	2300
TCGTCTGACC	TGCCTCAACC	TCCTGTCAAT	GCTGGCGGCG	GCTCTGGTGG
2310	2320	2330	2340	2350
TGGTTCTGGT	GGCGGCTCTG	AGGGTGGTGG	CTCTGAGGGT	GGCGGTTCTG
2360	2370	2380	2390	2400
AGGGTGGCGG	CTCTGAGGGA	GGCGGTTCCG	GTGGTGGCTC	TGGTTCCGGT
2410	2420	2430	2440	2450
GATTTTGATT	ATGAAAAGAT	GGCAAACGCT	AATAAGGGGG	CTATGACCGA
2460	2470	2480	2490	2500
AAATGCCGAT	GAAAACGCGC	TACAGTCTGA	CGCTAAAGGC	AAACTTGATT
2510	2520	2530	2540	2550
CTGTCGCTAC	TGATTACGGT	GCTGCTATCG	ATGGTTTCAT	TGGTGACGTT
2560	2570	2580	2590	2600
TCCGGCCTTG	CTAATGGTAA	TGGTGCTACT	GGTGATTTTG	CTGGCTCTAA
2610	2620	2630	2640	2650
TTCCCAAATG	GCTCAAGTCG	GTGACGGTGA	TAATTCACCT	TTAATGAATA
2660	2670	2680	2690	2700
ATTTCCGTCA	ATATTTACCT	TCCCTCCCTC	AATCGGTTGA	ATGTCGCCCT
2710	2720	2730	2740	2750
TTTGTCTTTA	GCGCTGGTAA	ACCATATGAA	TTTTCTATTG	ATTGTGACAA
2760	2770	2780	2790	2800
AATAAACTTA	TTCCGTGGTG	TCTTTGCGTT	TCTTTTATAT	GTTGCCACCT
2810	2820	2830	2840	2850
TTATGTATGT	ATTTTCTACG	TTTGCTAACA	TACTGCGTAA	TAAGGAGTCT
2860	2870	2880	2890	2900
TAATCATGCC	AGTTCTTTTG	GGTATTCCGT	TATTATTGCG	TTTCCTCGGT
2910	2920	2930	2940	2950
TTCCTTCTGG	TAACTTTGTT	CGGCTATCTG	CTTACTTTTC	TTAAAAAGGG
2960	2970	2980	2990	3000
CTTCGGTAAG	ATAGCTATTG	CTATTTCATT	GTTTCTTGCT	CTTATTATTG
3010	3020	3030	3040	3050
GGCTTAACTC	AATTCTTGTG	GGTTATCTCT	CTGATATTAG	CGCTCAATTA
3060	3070	3080	3090	3100
CCCTCTGACT	TTGTTCAGGG	TGTTCAGTTA	ATTCTCCCGT	CTAATGCGCT
3110	3120	3130	3140	3150
TCCCTGTTTT	TATGTTATTC	TCTCTGTAAA	GGCTGCTATT	TTCATTTTTG
3160	3170	3180	3190	3200
ACGTTAAACA	AAAAATCGTT	TCTTATTTGG	ATTGGGATAA	ATAATATGGC
3210	3220	3230	3240	3250
TGTTTATTTT	GTAACTGGCA	AATTAGGCTC	TGGAAAGACG	CTCGTTAGCG
3260	3270	3280	3290	3300
TTGGTAAGAT	TCAGGATAAA	ATTGTAGCTG	GGTGCAAAAT	AGCAACTAAT
3310	3320	3330	3340	3350
CTTGATTTAA	GGCTTCAAAA	CCTCCCGCAA	GTCGGGAGGT	TCGCTAAAAC
3360	3370	3380	3390	3400
GCCTCGCGTT	CTTAGAATAC	CGGATAAGCC	TTCTATATCT	GATTTGCTTG
3410	3420	3430	3440	3450
CTATTGGGCG	CGGTAATGAT	TCCTACGATG	AAAATAAAAA	CGGCTTGCTT
3460	3470	3480	3490	3500
GTTCTCGATG	AGTGCGGTAC	TTGGTTTAAT	ACCCGTTCTT	GGAATGATAA
3510	3520	3530	3540	3550
GGAAAGACAG	CCGATTATTG	ATTGGTTTCT	ACATGCTCGT	AAATTAGTAT
3560	3570	3580	3590	3600

FIGURE 9 (continued)

```
GGGATATTAT TTTTCTTGTT CAGGACTTAT CTATTGTTGA TAAACAGGCG
      3610       3620       3630       3640       3650
CGTTCTGCAT TAGCTGAACA TGTTGTTTAT TGTCGTCGTC TGGACAGAAT
      3660       3670       3680       3690       3700
TACTTTACCT TTTGTCGGTA CTTTATATTC TCTTATTACT GGCTCGAAAA
      3710       3720       3730       3740       3750
TGCCTCTGCC TAAATTACAT GTTGGCGTTG TTAAATATGG CGATTCTCAA
      3760       3770       3780       3790       3800
TTAAGCCCTA CTGTTGAGCG TTGGCTTTAT ACTGGTAAGA ATTTGTATAA
      3810       3820       3830       3840       3850
CGCATATGAT ACTAAACAGG CTTTTTCTAG TAATTATGAT TCCGGTGTTT
      3860       3870       3880       3890       3900
ATTCTTATTT AACGCCTTAT TTATCACACG GTCGGTATTT CAAACCATTA
      3910       3920       3930       3940       3950
AATTTAGGTC AGAAGATGAA ATTAACTAAA ATATATTTGA AAAAGTTTTC
      3960       3970       3980       3990       4000
TCGCGTTCTT TGTCTTGCGA TTGGATTTGC ATCAGCATTT ACATATAGTT
      4010       4020       4030       4040       4050
ATATAACCCA ACCTAAGCCG GAGGTTAAAA AGGTAGTCTC TCAGACCTAT
      4060       4070       4080       4090       4100
GATTTTGATA AATTCACTAT TGACTCTTCT CAGCGTCTTA ATCTAAGCTA
      4110       4120       4130       4140       4150
TCGCTATGTT TTCAAGGATT CTAAGGGAAA ATTAATTAAT AGCGACGATT
      4160       4170       4180       4190       4200
TACAGAAGCA AGGTTATTCA CTCACATATA TTGATTTATG TACTGTTTCC
      4210       4220       4230       4240       4250
ATTAAAAAAG GTAATTCAAA TGAAATTGTT AAATGTAATT AATTTTGTTT
      4260       4270       4280       4290       4300
TCTTGATGTT TGTTTCATCA TCTTCTTTTG CTCAGGTAAT TGAAATGAAT
      4310       4320       4330       4340       4350
AATTCGCCTC TGCGCGATTT TGTAACTTGG TATTCAAAGC AATCAGGCGA
      4360       4370       4380       4390       4400
ATCCGTTATT GTTTCTCCCG ATGTAAAAGG TACTGTTACT GTATATTCAT
      4410       4420       4430       4440       4450
CTGACGTTAA ACTTGAAAAT CTACGCAATT TCTTTATTTC TGTTTTACGT
      4460       4470       4480       4490       4500
GCTAATAATT TTGATATGGT TGGTTCAATT CCTTCCATAA TTCAGAAGTA
      4510       4520       4530       4540       4550
TAATCCAAAC AATCAGGTAT ATATTGATGA ATTGCCATCA TCTGATAATC
      4560       4570       4580       4590       4600
AGGAATATGA TGATAATTCC GCTCCTTCTG GTGGTTTCTT TGTTCCGCAA
      4610       4620       4630       4640       4650
AATGATAATG TTACTCAAAC TTTTAAAATT AATAACGTTC GGGCAAAGGA
      4660       4670       4680       4690       4700
TTTAATACGA GTTGTCGAAT TGTTTGTAAA GTCTAATACT TCTAAATCCT
      4710       4720       4730       4740       4750
CAAATGTATT ATCTATTGAC GGCTCTAATC TATTAGTTGT TAGTGCACCT
      4760       4770       4780       4790       4800
AAAGATATTT TAGATAACCT TCCTCAATTC CTTTCTACTG TTGATTTGCC
      4810       4820       4830       4840       4850
AACTGACCAG ATATTGATTG AGGGTTTGAT ATTTGAGGTT CAGCAAGGTG
      4860       4870       4880       4890       4900
ATGCTTTAGA TTTTTCATTT GCTGCTGGCT CTCAGCGTGG CACTGTTGCA
      4910       4920       4930       4940       4950
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FIGURE 9 (continued)

GGCGGTGTTA ATACTGACCG CCTCACCTCT GTTTTATCTT CTGCTGGTGG
4960 4970 4980 4990 5000
TTCGTTCGGT ATTTTTAATG GCGATGTTTT AGGGCTATCA GTTCGCGCAT
5010 5020 5030 5040 5050
TAAAGACTAA TAGCCATTCA AAAATATTGT CTGTGCCACG TATTCTTACG
5060 5070 5080 5090 5100
CTTTCAGGTC AGAAGGGTTC TATCTCTGTT GGCCAGAATG TCCCTTTTAT
5110 5120 5130 5140 5150
TACTGGTCGT GTGACTGGTG AATCTGCCAA TGTAAATAAT CCATTTCAGA
5160 5170 5180 5190 5200
CGATTGAGCG TCAAAATGTA GGTATTTCCA TGAGCGTTTT TCCTGTTGCA
5210 5220 5230 5240 5250
ATGGCTGGCG GTAATATTGT TCTGGATATT ACCAGCAAGG CCGATAGTTT
5260 5270 5280 5290 5300
GAGTTCTTCT ACTCAGGCAA GTGATGTTAT TACTAATCAA AGAAGTATTG
5310 5320 5330 5340 5350
CTACAACGGT TAATTTGCGT GATGGACAGA CTCTTTTACT CGGTGGCCTC
5360 5370 5380 5390 5400
ACTGATTATA AAAACACTTC TCAAGATTCT GGCGTACCGT TCCTGTCTAA
5410 5420 5430 5440 5450
AATCCCTTTA ATCGGCCTCC TGTTTAGCTC CCGCTCTGAT TCCAACGAGG
5460 5470 5480 5490 5500
AAAGCACGTT ATACGTGCTC GTCAAAGCAA CCATAGTACG CGCCCTGTAG
5510 5520 5530 5540 5550
CGGCGCATTA AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA
5560 5570 5580 5590 5600
CACTTGCCAG CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT
5610 5620 5630 5640 5650
CTCGCCACGT TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC
5660 5670 5680 5690 5700
TTTAGGGTTC CGATTTAGTG CTTTACGGCA CCTCGACCCC AAAAAACTTG
5710 5720 5730 5740 5750
ATTTGGGTGA TGGTTCACGT AGTGGGCCAT CGCCCTGATA GACGGTTTTT
5760 5770 5780 5790 5800
CGCCCTTTGA CGTTGGAGTC CACGTTCTTT AATAGTGGAC TCTTGTTCCA
5810 5820 5830 5840 5850
AACTGGAACA ACACTCAACC CTATCTCGGG CTATTCTTTT GATTTATAAG
5860 5870 5880 5890 5900
GGATTTTGCC GATTTCGGAA CCACCATCAA ACAGGATTTT CGCCTGCTGG
5910 5920 5930 5940 5950
GGCAAACCAG CGTGGACCGC TTGCTGCAAC TCTCTCAGGG CCAGGCGGTG
5960 5970 5980 5990 6000
AAGGGCAATC AGCTGTTGCC CGTCTCGCTG GTGAAAAGAA AAACCACCCT
6010 6020 6030 6040 6050
GGCGCCCAAT ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA
6060 6070 6080 6090 6100
TCCAGCTGGC ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA
6110 6120 6130 6140 6150
CGCAATTAAT GTGAGTTACC TCACTCATTA GGCACCCCAG GCTTTACACT
6160 6170 6180 6190 6200
TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT
6210 6220 6230 6240 6250
CACACAGGAA ACAGCTATGA CCATGATTAC GAATTCGAGC TCGCCCGGGG
6260 6270 6280 6290 6300

FIGURE 9 (continued)

ATCTGCCTGA	ATAGGTACGA	TTTACTAACT	GGAAGAGGCA	CTAAATGAAC
6310	6320	6330	6340	6350
ACGATTAACA	TCGCTAAGAA	CGACTTCTCT	GACATCGAAC	TGGCTGCTAT
6360	6370	6380	6390	6400
CCCGTTCAAC	ACTCTGGCTG	ACCATTACGG	TGAGCGTTTA	GCTCGCGAAC
6410	6420	6430	6440	6450
AGTTGGCCCT	TGAGCATGAG	TCTTACGAGA	TGGGTGAAGC	ACGCTTCCGC
6460	6470	6480	6490	6500
AAGATGTTTG	AGCGTCAACT	TAAAGCTGGT	GAGGTTGCGG	ATAACGCTGC
6510	6520	6530	6540	6550
CGCCAAGCCT	CTCATCACTA	CCCTACTCCC	TAAGATGATT	GCACGCATCA
6560	6570	6580	6590	6600
ACGACTGGTT	TGAGGAAGTG	AAAGCTAAGC	GCGGCAAGCG	CCCGACAGCC
6610	6620	6630	6640	6650
TTCCAGTTCC	TGCAAGAAAT	CAAGCCGGAA	GCCGTAGCGT	ACATCACCAT
6660	6670	6680	6690	6700
TAAGACCACT	CTGGCTTGCC	TAACCAGTGC	TGACAATACA	ACCGTTCAGG
6710	6720	6730	6740	6750
CTGTAGCAAG	CGCAATCGGT	CGGGCCATTG	AGGACGAGGC	TCGCTTCGGT
6760	6770	6780	6790	6800
CGTATCCGTG	ACCTTGAAGC	TAAGCACTTC	AAGAAAAACG	TTGAGGAACA
6810	6820	6830	6840	6850
ACTCAACAAG	CGCGTAGGGC	ACGTCTACAA	GAAAGCATTT	ATGCAAGTTG
6860	6870	6880	6890	6900
TCGAGGCTGA	CATGCTCTCT	AAGGGTCTAC	TCGGTGGCGA	GGCGTGGTCT
6910	6920	6930	6940	6950
TCGTGGCATA	AGGAAGACTC	TATTCATGTA	GGAGTACGCT	GCATCGAGAT
6960	6970	6980	6990	7000
GCTCATTGAG	TCAACCGGAA	TGGTTAGCTT	ACACCGCCAA	AATGCTGGCG
7010	7020	7030	7040	7050
TAGTAGGTCA	AGACTCTGAG	ACTATCGAAC	TCGCACCTGA	ATACGCTGAG
7060	7070	7080	7090	7100
GCTATCGCAA	CCCGTGCAGG	TGCGCTGGCT	GGCATCTCTC	CGATGTTCCA
7110	7120	7130	7140	7150
ACCTTGCGTA	GTTCCTCCTA	AGCCGTGGAC	TGGCATTACT	GGTGGTGGCT
7160	7170	7180	7190	7200
ATTGGGCTAA	CGGTCGTCGT	CCTCTGGCGC	TGGTGCGTAC	TCACAGTAAG
7210	7220	7230	7240	7250
AAAGCACTGA	TGCGCTACGA	AGACGTTTAC	ATGCCTGAGG	TGTACAAAGC
7260	7270	7280	7290	7300
GATTAACATT	GCGCAAAACA	CCGCATGGAA	AATCAACAAG	AAAGTCCTAG
7310	7320	7330	7340	7350
CGGTCGCCAA	CGTAATCACC	AAGTGGAAGC	ATTGTCCGGT	CGAGGACATC
7360	7370	7380	7390	7400
CCTGCGATTG	AGCGTGAAGA	ACTCCCGATG	AAACCGGAAG	ACATCGACAT
7410	7420	7430	7440	7450
GAATCCTGAG	GCTCTCACCG	CGTGGAAACG	TGCTGCCGCT	GCTGTGTACC
7460	7470	7480	7490	7500
GCAAGGACAA	GGCTCGCAAG	TCTCGCCGTA	TCAGCCTTGA	GTTCATGCTT
7510	7520	7530	7540	7550
GAGCAAGCCA	ATAAGTTTGC	TAACCATAAG	GCCATCTGGT	TCCCTTACAA
7560	7570	7580	7590	7600
CATGGACTGG	CGCGGTCGTG	TTTACGCTGT	GTCAATGTTC	AACCCGCAAG
7610	7620	7630	7640	7650

FIGURE 9 (continued)

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GTAACGATAT GACCAAAGGA CTGCTTACGC TGGCGAAAGG TAAACCAATC
      7660       7670       7680       7690       7700
GGTAAGGAAG GTTACTACTG GCTGAAAATC CACGGTGCAA ACTGTGCGGG
      7710       7720       7730       7740       7750
TGTCGATAAG GTTCCGTTCC CTGAGCGCAT CAAGTTCATT GAGGAAAACC
      7760       7770       7780       7790       7800
ACGAGAACAT CATGGCTTGC GCTAAGTCTC CACTGGAGAA CACTTGGTGG
      7810       7820       7830       7840       7850
GCTGAGCAAG ATTCTCCGTT CTGCTTCCTT GCGTTCTGCT TTGAGTACGC
      7860       7870       7880       7890       7900
TGGGGTACAG CACCACGGCC TGAGCTATAA CTGCTCCCTT CCGCTGGCGT
      7910       7920       7930       7940       7950
TTGACGGGTC TTGCTCTGGC ATCCAGCACT TCTCCGCGAT GCTCCGAGAT
      7960       7970       7980       7990       8000
GAGGTAGGTG GTCGCGCGGT TAACTTGCTT CCTAGTGAAA CCGTTCAGGA
      8010       8020       8030       8040       8050
CATCTACGGG ATTGTTGCTA AGAAAGTCAA CGAGATTCTA CAAGCAGACG
      8060       8070       8080       8090       8100
CAATCAATGG GACCGATAAC GAAGTAGTTA CCGTGACCGA TGAGAACACT
      8110       8120       8130       8140       8150
GGTGAAATCT CTGAGAAAGT CAAGCTGGGC ACTAAGGCAC TGGCTGGTCA
      8160       8170       8180       8190       8200
ATGGCTGGCT TACGGTGTTA CTCGCAGTGT GACTAAGCGT TCAGTCATGA
      8210       8220       8230       8240       8250
CGCTGGCTTA CGGGTCCAAA GAGTTCGGCT TCCGTCAACA AGTGCTGGAA
      8260       8270       8280       8290       8300
GATACCATTC AGCCAGCTAT TGATTCCGGC AAGGGTCTGA TGTTCACTCA
      8310       8320       8330       8340       8350
GCCGAATCAG GCTGCTGGAT ACATGGCTAA GCTGATTTGG GAATCTGTGA
      8360       8370       8380       8390       8400
GCGTGACGGT GGTAGCTGCG GTTGAAGCAA TGAACTGGCT TAAGTCTGCT
      8410       8420       8430       8440       8450
GCTAAGCTGC TGGCTGCTGA GGTCAAAGAT AAGAAGACTG GAGAGATTCT
      8460       8470       8480       8490       8500
TCGCAAGCGT TGCGCTGTGC ATTGGGTAAC TCCTGATGGT TTCCCTGTGT
      8510       8520       8530       8540       8550
GGCAGGAATA CAAGAAGCCT ATTCAGACGC GCTTGAACCT GATGTTCCTC
      8560       8570       8580       8590       8600
GGTCAGTTCC GCTTACAGCC TACCATTAAC ACCAACAAAG ATAGCGAGAT
      8610       8620       8630       8640       8650
TGATGCACAC AAACAGGAGT CTGGTATCGC TCCTAACTTT GTACACAGCC
      8660       8670       8680       8690       8700
AAGACGGTAG CCACCTTCGT AAGACTGTAG TGTGGGCACA CGAGAAGTAC
      8710       8720       8730       8740       8750
GGAATCGAAT CTTTTGCACT GATTCACGAC TCCTTCGGTA CCATTCCGGC
      8760       8770       8780       8790       8800
TGACGCTGCG AACCTGTTCA AAGCAGTGCG CGAAACTATG GTTGACACAT
      8810       8820       8830       8840       8850
ATGAGTCTTG TGATGTACTG GCTGATTTCT ACGACCAGTT CGCTGACCAG
      8860       8870       8880       8890       8900
TTGCACGAGT CTCAATTGGA CAAAATGCCA GCACTTCCGG CTAAAGGTAA
      8910       8920       8930       8940       8950
CTTGAACCTC CGTGACATCT TAGAGTCGGA CTTCGCGTTC GCGTAACGCC
      8960       8970       8980       8990       9000
```

FIGURE 9 (continued)

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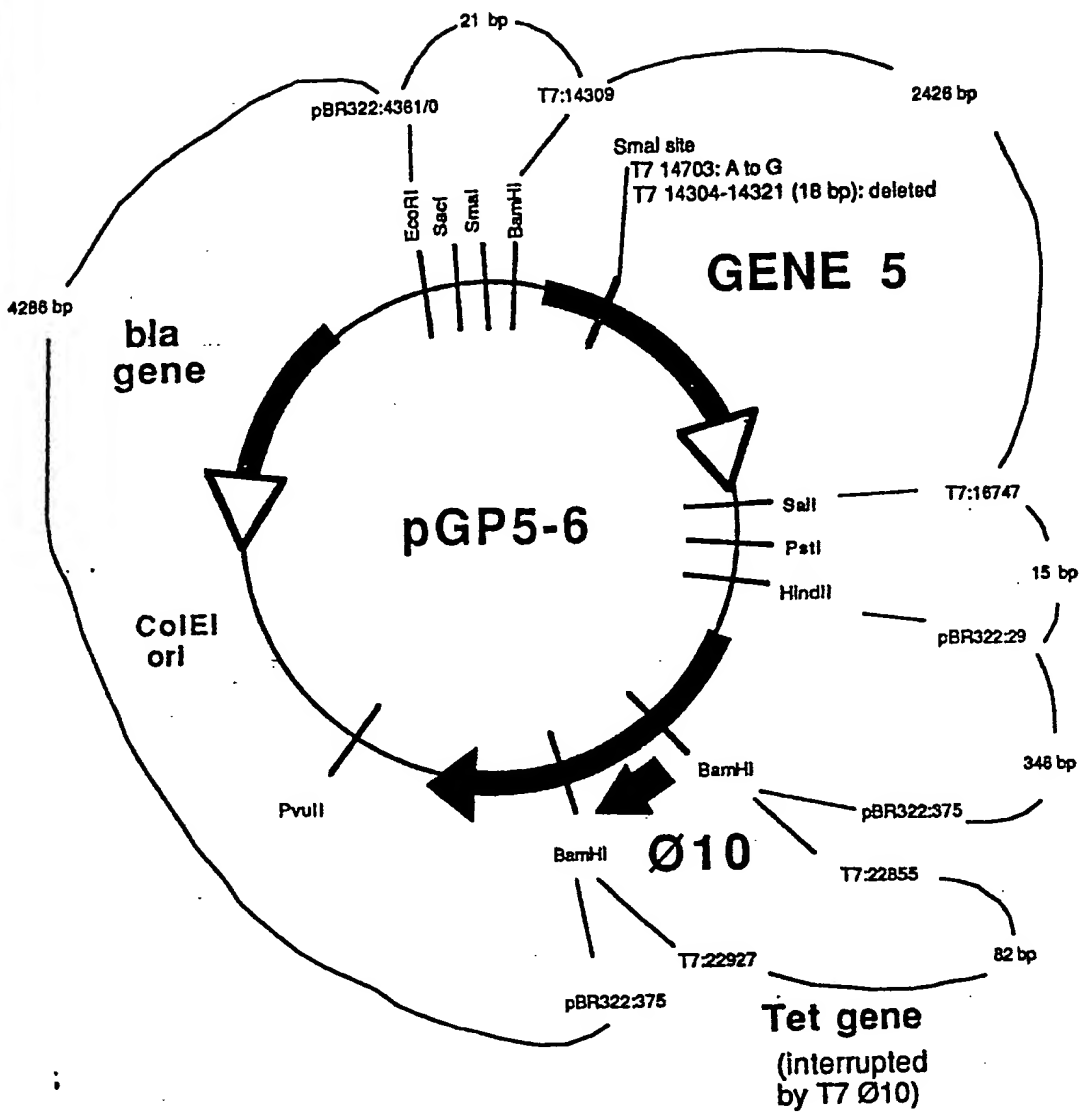
AAATCAATAC GACCCGGATC GGTCGACCTG CAGCCCAAGC TTGGCACTGG
      9010       9020       9030       9040       9050
CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT TACCCAACTT
      9060       9070       9080       9090       9100
AATCGCCTTG CAGCACATCC CCCCTTCGCC AGCTGGCGTA ATAGCGAAGA
      9110       9120       9130       9140       9150
GGCCCGCACC GATCGCCCTT CCCAACAGTT GCGTAGCCTG AATGGCGAAT
      9160       9170       9180       9190       9200
GGCGCTTTGC CTGGTTTCCG GCACCAGAAG CGGTGCCGGA AAGCTGGCTG
      9210       9220       9230       9240       9250
GAGTGCGATC TTCCTGAGGC CGAQACNGTC GTCGTCCCCT CAAACTGGCA
      9260       9270       9280       9290       9300
GATGCACGGT TACGATGCGC CCATCTACAC CAACGTAACC TATCCCATTA
      9310       9320       9330       9340       9350
CGGTCAATCC GCCGTTTGTT CCCACGGAGA ATCCGACGGG TTGTTACTCG
      9360       9370       9380       9390       9400
CTCACATTTA ATGTTGATGA AAGCTGGCTA CAGGAAGGCC AGACGCGAAT
      9410       9420       9430       9440       9450
TATTTTTGAT GGCGTTCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA
      9460       9470       9480       9490       9500
ATTTAACGCG AATTTTAACA AAATATTAAC GTTTACAATT TAAATATTTG
      9510       9520       9530       9540       9550
CTTATACAAT CTTCCTGTTT TTGGGGCTTT TCTGATTATC AACCGGGGTA
      9560       9570       9580       9590       9600
CATATGATTG ACATGCTAGT TTTACGATTA CCGTTCATCG ATTCTCTTGT
      9610       9620       9630       9640       9650
TTGCTCCAGA CTCTCAGGCA ATGACCTGAT AGCCTTTGTA GATCTCTCAA
      9660       9670       9680       9690       9700
AAATAGCTAC CCTCTCCGGC ATGAATTTAT CAGCTAGAAC GGTTGAATAT
      9710       9720       9730       9740       9750
CATATTGATG GTGATTTGAC TGTCTCCGGC CTTTCTCACC CTTTTGAATC
      9760       9770       9780       9790       9800
TTTACCTACA CATTACTCAG GCATTGCATT TAAAATATAT GAGGGTTCTA
      9810       9820       9830       9840       9850
AAAATTTTTA TCCTTGCGTT GAAATAAAGG CTTCTCCCGC AAAAGTATTA
      9860       9870       9880       9890       9900
CAGGGTCATA ATGTTTTTGG TACAACCGAT TTAGCTTTAT GCTCTGAGGC
      9910       9920       9930       9940       9950
TTTATTGCTT AATTTTGCTA ATTCTTTGCC TTGCCTGTAT GATTTATTGG

ATGTT
```

FIGURE 10



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pBR322:4361/0
T7:14309
2426 bp
Smal site
T7 14703: A to G
T7 14304-14321 (18 bp): deleted
EcoRI
SacI
SmaI
BamHI
GENE 5
4286 bp
bla
gene
pGP5-6
SalI
PstI
HindII
T7:16747
15 bp
pBR322:29
ColEI
ori
348 bp
BamHI
pBR322:375
PvuII
BamHI
Ø10
T7:22855
82 bp
T7:22927
pBR322:375
Tet gene
(interrupted
by T7 Ø10)